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(71) Applicant *(for all designated States except US)*: NEURALSTEM BIOPHARMACEUTICALS [US/US]; Building 335, Paint Branch Drive, College Park, MD 20742 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: JOHE, Karl, K. [US/US]; 10841 Stanmore Drive, Potomac, MD 20854 (US).

(74) Agent: PICCIANO, Dante, J.; Bell, Boyd & Lloyd, P.O. Box 65331, Washington, DC 20035-5331 (US).

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(54) Title: ISOLATION, PROPAGATION, AND DIRECTED DIFFERENTIATION OF STEM CELLS FROM CENTRAL NERVOUS SYSTEM OF MAMMALS

(57) Abstract

The present invention reveals an *in vitro* procedure by which an homogeneous population of multipotential precursor cells from mammalian embryonic neuroepithelium (CNS stem cells) can be expanded up to 10^9 fold in culture while maintaining their multipotential capacity to differentiate into neurons, oligodendrocytes, and astrocytes. Chemically defined conditions are presented that enable a large number of neurons, up to 50 % of the expanded cells, to be derived from the stem cells. In addition, four factors -- PDGF, CNTF, LIF and T3 -- have been identified, which, individually, generate significantly higher proportion of neurons, astrocytes, or oligodendrocytes. These defined procedures permit a large-scale preparation of the mammalian CNS stem cells, neurons, astrocytes, and oligodendrocytes under chemically defined conditions with efficiency and control. The present invention also reveals *in vitro* cultures of region-specific, terminally differentiated, mature neurons derived from cultures of mammalian multipotential CNS stem cells and an *in vitro* procedure by which the differentiated neurons may be generated.

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ISOLATION, PROPAGATION, AND DIRECTED DIFFERENTIATION OF STEM CELLS FROM CENTRAL NERVOUS SYSTEM OF MAMMALS

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention relates to a technology where stem cells from embryonic and adult brain are isolated, propagated, and differentiated efficiently in culture to generate large numbers of nerve cells. This technology, for the first time, enables one to generate large numbers of many different kinds of neurons found in a normal brain and provides a new foundation for gene therapy, cell therapy, novel growth factor screening, and drug screening for nervous system disorders.

2. DESCRIPTION OF THE RELATED ART

The brain is composed of highly diverse nerve cell types making specific interconnections and, once destroyed, the nerve cells (neurons) do not regenerate. In addition, the brain is protected by a blood-brain barrier that effectively blocks the flow of large molecules into the brain, rendering peripheral injection of potential growth factor drugs ineffective. Thus, a major challenge currently facing the biotechnology industry is to find an efficient mechanism for delivering

potential gene therapy products directly into the brain in order to treat nervous system disorders.

Moreover, for a degenerative disease like Parkinson's, the most comprehensive approach to regain a lost neural function may be to replace the damaged cells with healthy cells, rather than just a single gene product. Thus, current and future success of gene therapy and cell therapy depends upon development of suitable cells that can (1) carry a healthy copy of a disease gene (i.e., a normal gene), (2) be transplanted into the brain, and (3) be integrated into the host's neural network. This development ideally requires cells of neuronal origin that (1) proliferate in culture to a large number, (2) are amenable to various methods of gene transfer, and (3) integrate and behave as the cells of a normal brain. However, there have been no such cells for therapeutic purposes since neurons do not divide and therefore cannot be propagated in culture.

As alternatives, various transformed cells of neural and non-neural origins such as glias, fibroblasts, and even muscle cells, which can be proliferated in culture, have been used as possible vehicles for delivering a gene of interest into brain cells. However, such cells do not and cannot be expected to provide neuronal functions. Another alternative approach has been

to force a neural cell of unknown origin to divide in culture by genetically modifying some of its properties, while still retaining some of its ability to become and function as a neuron. Although some "immortalized" cells can display certain features of a neuron, it is unclear whether these altered cells are truly a viable alternative for clinical purposes.

A developing fetal brain contains all of the cells germinal to the cells of an adult brain as well as all of the programs necessary to orchestrate them toward the final network of neurons. At early stages of development, the nervous system is populated by germinal cells from which all other cells, mainly neurons, astrocytes, and oligodendrocytes, derive during subsequent stages of development. Clearly, such germinal cells that are precursors of the normal brain development would be ideal for all gene-based and cell-based therapies if these germinal cells could be isolated, propagated, and differentiated into mature cell types.

The usefulness of the isolated primary cells for both basic research and for therapeutic application depends upon the extent to which the isolated cells resemble those in the brain. Just how many different kinds of precursor cells there are in the developing brain is unknown. However,

several distinct cell types may exist:

a precursor to neuron only ("committed neuronal progenitor" or "neuroblast"),

a precursor to oligodendrocyte only ("oligodendroblast"),

a precursor to astrocyte only ("astroblast"),

a bipotential precursor that can become either neuron or oligodendrocyte, neuron or astrocyte, and oligodendrocyte or astrocyte, and

a multipotential precursor that maintains the capacity to differentiate into any one of the three cell types.

Fate mapping analysis and transplantation studies *in vivo* have shown that different neuronal types and non-neuronal cells can be derived from the same precursor cells[1-5]. *In vitro* analyses have also suggested that multipotential cells are present in the developing brain[6,7]. Lineage analysis alone, however, does not directly identify the multipotential cells; nor does it define the mechanisms that drive them to different fates. Precursor cells from the central nervous system (CNS) have been expanded *in vitro* and differentiation into neurons and glia has been observed[8-12] and, as detailed below, markedly different cell types have been obtained even when the culture conditions used were seemingly the same.

Because of the current lack of understanding of histogenesis during brain development, many investigators have used various terms loosely to describe the cells that they have studied, e.g., neuronal progenitor, neural precursor, neuroepithelial precursor, multipotential stem cell, etc. Thus, the nature of the cells so far described in the literature and culture conditions for obtaining them can only be compared to each other by their reported differentiation capacity. The entire subject of the isolation, characterization, and use of stem cells from the CNS has recently been reviewed [33,34,38].

In summary, conditions have not been found to date, despite many reports, to successfully identify, propagate, and differentiate multipotential stem cells. A useful compilation of studies reporting culture of CNS precursor cells is found in Table 3, p. 172, of a recent review[34] and further extended below.

Vicario-Abejon, C., Johe, K., Hazel, T., Collazo, D. & McKay, R., Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons, *Neuron* 15, 105-114 (1995)[12].

Cells expanded by Vicario-Abejon et al. are significantly different from those described in the present invention although the starting tissue (embryonic hippocampus), the mitogen (basic fibroblast growth factor, bFGF), and the basal

medium (N2) are similar in both reports. Almost all of the cells expanded by Vicario-Abejon et al. failed to differentiate into any cell types but died in the absence of bFGF (as stated in the paper, pg. 106). This is also reflected in Fig. 3 of the paper where the number of MAP2 positive neurons is exceedingly low (50-100 cells out of an initial cell number of approximately 80,000 per well; i.e., far less than 1% in all reported conditions). Thus, differences in culture conditions, subtle as they may be, can yield cells with significantly different properties and this is, in fact, consistent with the main observation of the present invention that the extracellular environment can shift the developmental properties of the CNS stem cells.

Vicario-Abejon et al. used the following culture conditions which differ from the those described in the present invention:

1. Used enzymatic dissociation, 0.1-0.25% trypsin + 0.4% DNAse I for the initial tissue dissociation as well as subsequent passaging. In the present invention, enzymatic dissociation effectively causes proteolyses of FGF receptors and causes cells to become unresponsive to bFGF and leads to differentiation.

2. Used 10% fetal bovine serum to stop the trypsin activity and to prime the cells from 4

hours to overnight before switching to serum free medium. In the present invention, serum even at less than 1% concentration shifts stem cells to astrocytic fate.

3. Cells were seeded at much higher density of 45,000 cells per cm^2 and then grown to confluence before passaging by trypsin and serum. In the present invention, high cell density inhibits proliferation and causes spontaneous differentiation even in the presence of bFGF.

4. bFGF was given only intermittently every 2-3 days, and at 5 ng/ml, less than the optimal concentration disclosed in the present invention. This condition leads to partial differentiation of cells and subsequent heterogeneity of cell types in culture.

5. Basal medium consisting of "N2" components consisted of 5 ng/ml insulin, less than the optimal concentration disclosed in the present invention.

Ray, J., Peterson, D., Schinstine, M. & Gage, F., Proliferation, differentiation, and long-term culture of primary hippocampal neurons, *Proc. Natl. Acad. Sci. USA* **90**, 3602-3606 (1993)[10].

This study used culture conditions that are very similar to those described by Vicario-Abejon et al.--bFGF as the primary mitogen, serum-free medium, and E16 hippocampus. However, it reports isolation and expansion of a precursor population (neuroblasts) quite different from the cells of

Vicario-Abejon et al. (undefined) as well as the multipotential stem cells described in the present invention. The reported cells had the following properties which markedly contrast from those of CNS stem cells:

1. The expanded cells under the reported condition are mitotic neurons with antigenic expressions of neurofilament, nestin, neuron-specific enolase, galactocerebroside, and MAP2 (Table I, p. 3604). The expanding CNS stem cells reported in the present invention express nestin, only, are negative for the above antigens, and are, therefore, a molecularly distinct population of cells from those described by Ray et al.

2. Ultrastructural analysis of the expanded cells in culture "demonstrated their histotypic neuronal morphology". The expanding CNS stem cells exhibit entirely different, non-neuronal morphology.

3. The mitotic "neurons" had a doubling time of 4 days and could be passaged and grown as continuous cell lines. The CNS stem cells double at every 20-24 hours and exhibit a characteristic regression of mitotic and differentiative capacity over time so that they cannot be maintained as stable cell lines indefinitely.

4. The culture system by Ray et al. generates

"nearly pure neuronal cell cultures". The culture system in the present invention generates multipotential stem cells that can differentiate into all three major cell types of the brain, i.e., neurons, oligodendrocytes, and astrocytes.

Ray et al. used the following culture conditions which differ from those of the present invention.

1. Embryonic hippocampi were mechanically triturated without the use of an enzyme; however, cells were plated approximately 100,000 cells per cm^2, optimal for neuronal survival, but almost 10 times higher cell density than optimal for expansion of CNS stem cells.

2. bFGF was given at 20 ng/ml, intermittently, at every 3-4 days.

3. Basal "N2" medium contained 5 μg/ml insulin, less than optimal. Medium change was also prolonged at every 3-4 days.

4. Cells were passaged by using trypsin.

In conclusion, even seemingly small differences in culture conditions can result in isolation of vastly different cell types.

<u>Ray, J. and Gage, F.H., Spinal cord neuroblasts proliferate in response to basic fibroblast growth factor, *J. Neurosci.* **14**, 3548-3564 (1994)[39]</u>.

Ray and Gage report isolation and propagation of cells "that have already committed to a neuronal pathway are and expressing neuronal

phenotypes (neuroblasts)" from spinal cord using bFGF. Again, although the primary mitogen is bFGF, their culture conditions are different and obtained cells markedly different from CNS stem cells.

1. E14-E16 spinal cord was used, a much later stage of development than optimal for stem cells.

2. The tissue was dissociated enzymatically by papain and DNase.

3. Initial plating was done in 10% fetal bovine serum.

4. There was a preliminary enrichment for a non-adherent cell population.

5. There was intermittent medium change and bFGF supplement, every 3-4 days.

Gage, F.H., Coates, P.W., Palmer, T.D., Kuhn, H.G., Fisher, L.J., Suhonen, J.O., Peterson, D.A., Suhr, S.T. & Ray, J., Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain, *Proc. Natl. Acad. Sci. USA* **92**, 11879-11883 (1995)[35].

Gage et al. report isolation, propagation, and transplantation of cells from adult hippocampus. These mixtures of cells were maintained in culture for one year through multiple passages. 80% of them exhibit rather unusual properties such as co-expressing glial and neuronal antigens while remaining mitotic. These properties are not exhibited by stem cells isolated from the adult striatal subventricular zone.

Again, using bFGF as a primary mitogen, the

authors derived markedly different cells than CNS stem cells reported in the present invention.

Gritti, A. et al., Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor, *J. Neurosci.* 16, 1091-1100 (1996)[40].

These authors report isolation and propagation of multipotential stem cells from the subventricular zone of adult brain by using bFGF. A significant difference in culture conditions used by Gritti et al. is that the cells are propagated as aggregated spheres without attachment to plate surface. Culture conditions by Gritti et al. require this aggregation of cells into spheres, using either bFGF or epidermal growth factor (EGF), as an essential step for propagating multipotential cells. This aggregation step alone essentially distinguishes the reported culture system from that of the present invention. The aggregation promotes undefined cell-cell interactions and results in uncontrollable differentiation/fate-shifts and overall in much less expansion and differentiation. Furthermore, this culture system and the result obtained by Gritti et al. are limited to adult brain where extremely small number of cells were obtained (10^5 cells per brain) and have not been extended to various regions of embryonic brain.

The procedure in the present invention permits

propagation of stem cells throughout the developing CNS as well as the striatum of the adult brain. It also uses adherent culture and actively avoids cell-cell contact and high cell density. As a result, it permits much more efficient expansion of the cells in an undifferentiated multipotential state and much more precise and efficient control over differentiation of the expanded cells.

Reynolds, B. & Weiss, S., Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system, *Science* 255, 1707-1710 (1992)[15].

Reynolds, B., Tetzlaff, W. & Weiss, S., A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes, *J. Neurosci.* 12, 4565-4574 (1992)[9].

Vescovi, A.L., Reynolds, B.A., Fraser, D.D., and Weiss, S., bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/ astroglial) EGF-generated CNS progenitor cells, *Neuron* 11, 951-966 (1993)[41].

These three studies describe the original sphere cultures of neural precursor cells from adult and embryonic brain using EGF (epidermal growth factor). The expanded cells differentiate into neurons and astrocytes, but not into oligodendrocytes, and thus are thought to be a bipotential population, rather than multipotential. Another distinguishing property of the cells is that they respond only to EGF and not to bFGF in particular, whereas CNS stem cells respond similarly to both EGF and bFGF. Again, the sphere culture conditions are not comparable

to those employed in the present invention because they require cell aggregation in which many additional undefined interactions are expected to occur.

<u>Ahmed, S., Reynolds, B.A., and Weiss, S., BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors, *J. Neurosci.* **15**, 5765-5778 (1995)</u>[42].

This paper reports the effects of brain-derived growth factor (BDNF) on sphere cultures of embryonic neural precursor cells propagated with EGF. There is no further enhancement of the culture system per se.

<u>Svendsen, C.N., Fawcett, J.W., Bentlage, C. & Dunnett, S.B., Increased survival of rat EGF-generated CNS precursor cells using B27 supplemented medium, *Exp. Brain Res.* **102**, 407-414 (1995)</u>[36].

This study utilizes the sphere culture with EGF as described above to test a commercially available medium supplement called "B27". The study simply reports that use of B27 enhances <u>cell</u> survival (not neuronal survival) in a mixed culture containing neurons, astrocytes, and oligodendrocytes.

<u>Kilpatrick, T.J. and Bartlett, P.F., Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation, *Neuron* **10**, 255-265 (1993)</u>[43].

The authors report existence of multipotential precursor cells in E10 mouse telencephalon by culturing single cells from the brain in bFGF plus serum. The results were based on 700 cells

expanded clonally for 10 days, some of which, when differentiated in the presence of bFGF, serum, and astrocyte conditioned medium, could give rise to neurons. There was no mass expansion of the cells.

Kilpatrick, T.J. and Bartlett, P.F., Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF, *J. Neurosci.* 15, 3653-3661 (1995)[44].

The authors utilize the clonal culture system reported in the above-described reference[43] to test mitogenic efficacy of bFGF and EGF on cortical cells from E10 and E17 embryos. Again, the culture condition applies strictly to microculture in serum containing medium to demonstrate existence of different precursor cells in developing brain. There is no mass expansion, long-term culture, or systematic differentiation protocol.

Baetge, E.E., Neural stem cells for CNS transplantation, *Ann. N.Y. Acad. Sci.* 695, 285 (1993)[45].

This is a brief review paper summarizing various studies directed to isolating precursor cells and their derivatives in culture. It is somewhat outdated and most of the relevant original studies cited have been discussed above.

Bartlett, P.F. et al., Regulation of neural precursor differentiation in the embryonic and adult forebrain, *Clin. Exp. Pharm. Physiol.* 22, 559-562 (1995)[46].

This is also a brief review paper summarizing

mostly previous works from the authors' laboratory in regard to their microculture studies where differentiation potentials of certain clones of precursors are tested in the presence of acidic FGF (aFGF), bFGF, serum, and/or astrocyte conditioned medium.

In addition, Sabate et al.[32] reported the culturing of a human neural progenitor with undefined differentiation capacity. Davis and Temple[6] demonstrated the existence of multipotential stem cells in cortex by co-culturing with epithelial cells for short term (less than 100 cells altogether).

However, cell differentiation could not be controlled in any of the reported studies which precluded analysis of their lineage relations and the mechanisms regulating fate choice.

The present invention provides a method for efficiently propagating the undifferentiated germinal cells, i.e., stem cells of the central nervous system (CNS), in culture and defines conditions to effectively turn the undifferentiated cells into mature cell types. These undifferentiated cells or "CNS stem cells" display the multipotential capacity to differentiate into all three major cell types of a mature brain -- neurons, astrocytes, and oligodendrocytes. Moreover, the same culture

conditions enable isolation, expansion, and differentiation of equivalent multipotential cells from the adult brain.

Since the initial disclosure, additional reports have appeared. Most recent research on and use of CNS stem cells and neural progenitors have been further reviewed[47-52]. In addition, Reynolds and Weiss[53] reported that embryonic striatal progenitors generated as spheres using EGF were able to differentiate into all three cell types including oligodendrocytes, astrocytes, and neurons. The frequency of EGF-responsive cells was limited to only 1% of the initial primary culture. Subcloning to establish self-renewal was questionable since up to 500 cells/well were used to generate the secondary "clones". Differentiation of the cells was induced by incorporating 1% serum in the medium. However, no data demonstrating all three cell types were presented from single-cell derived clones.

Weiss et al.[54] reported that multipotential CNS stem cells could be isolated from adult spinal cord and third and fourth ventricles by using a combination of EGF and bFGF but not with either alone.

Svendsen et al.[55] reported that neural precursor cells isolated from striatum and mesencephalon of 16 day old rat embryos (E16),

when grafted into lesioned adult rat brains, failed to differentiate into neurons. They also reported that EGF-generated mesencephalon cells but not striatal cells differentiated into tyrosine hydroxylase (TH)-positive neurons, albeit in very low number (0.002%). There were no characterization of cells *in vitro* to ensure that the primary culture used contained no post-mitotic neurons carrying over from the tissue, especially given that the result could only be obtained with E16 tissue when most TH cells are already born.

Schinstine and Iacovitti[56] reported that some of the astrocytes derived from EGF-generated neural precursor cells expressed neuronal antigens such as tau and MAP2. Qian et al.[57] reported that different concentrations of bFGF proliferate stem-like cells of E10 mouse cortex with varying differentiation potentials ranging from only neuronal to multipotential.

Palmer et al.[65] reported that multipotential CNS stem cells could be isolated from adult rat hippocampus. 84% of the cells they expanded, however, co-expressed MAP2c and O4, immature neuronal and oligodendroglial markers. Only 0.2% were MAP2ab positive and less than 0.01% were positive for other neuronal markers such as tau and neurofilament 200. Such properties are quite different from the properties described in the

Examples in the present application.

Finley et al.[66] reported that the mouse embryonic carcinoma cells line, P19, can form neuronal polarity and be eletrophysiologically active when induced by retinoic acid and serum. Strubing et al.[67] reported that embryonic stem cells grown in serum-containing medium could differentiate into electrophysiologically active neurons *in vitro*. Okabe et al.[68] also reported differentiation of some of embryonic stem cells into neurons *in vitro*.

Gritti et al.[40] reported that multipotential stem cells could be isolated from adult mouse subependyme by EGF and bFGF, which when differentiated, could be eletrophysiologically active and express GABA-, gluatamate-, and ChAT-immunoreactivities, but not others. The frequency of such neurons, however, was not documented and thus it is difficult to ascertain how efficient neuronal maturation was. Moreover, these neuronal phenotypes derived from dividing stem cells were not directly demonstrated by BrdU labeling. This is particularly relevant since aggregate cultures are extremely prone to be contaminated by primary neurons from the tissue, which carry over for several passages. Weiss et al.[49], in fact, stated that only GABA-positive cells could be obtained from their cultures. Most of the GABA-positive

cells may be oligodendrocytes.

Feldman et al.[69] reported electrophysiological studies of EGF-generated rat neural precursors. They found that most, if not all, electrophysiologically active cells are in fact non-neuronal, and that glial cells do contain voltage-sensitive Na channels that evoke action potential-like conductances.

Results such as these illustrate that identifying CNS stem cells, defining conditions that stably maintain CNS stem cell properties for long-term, and controlling their differentiation into mature cell types are neither obvious nor predictable to those skilled in this art.

SUMMARY OF THE INVENTION

The present invention discloses an *in vitro* culture of stem cells of the central nervous system of a mammal, a method for the *in vitro* culture of the stem cells, and a method for the differentiation of the stem cells.

In the *in vitro* culture of the stem cells of the central nervous system of a mammal, the stem cells maintain the multipotential capacity to differentiate into neurons, astrocytes, and oligodendrocytes. The stem cells can be derived from central nervous system tissue from a human, fetus or adult. Furthermore, the central nervous

system tissue may be hippocampus, cerebral cortex, striatum, septum, diencephalon, mesencephalon, hindbrain, or spinal cord.

Furthermore, the stem cells can differentiate to mature neurons exhibiting axon-dendrite polarity, synaptic terminals, and localization of proteins involved in synaptogenesis and synaptic activity including neurotransmitter receptors, transporters, and processing enzymes. In addition, the stem cells retain their capacity to generate subtypes of neurons having molecular differences among the subtypes.

In the method for the *in vitro* culture of the stem cells, where the stem cells maintain the multipotential capacity to differentiate into neurons, astrocytes, and oligodendrocytes, cells from the central nervous system are:

a) dissociated by mechanical trituration;

b) plated at the optimal initial density of 1×10^6 cells (from hippocampus and septum) or 1.5×10^6 cells (from other CNS regions) per 10 cm plate precoated with poly-ornithine and fibronectin;

c) cultured in the complete absence of serum;

d) supplied daily with a growth factor selected from the group consisting of

i) basic fibroblast growth factor

(bFGF) at a concentration of at least 10 ng/ml,

ii) EGF at a concentration of at least 10 ng/ml,

iii) TGF-alpha at a concentration of at least 10 ng/ml, and

iv) acidic FGF (aFGF) at a concentration of at least 10 ng/ml plus 1 μg/ml heparin;

e) replaced 100% of culture medium every two days with fresh medium;

f) passaged at every 4 days after plating by treating the cultured cells with saline solution and scraping the cells from the plate; and

g) replated passaged cells at 0.5×10^6 cells per 10 cm plate precoated with poly-ornithine and fibronectin.

The method is applicable with stems cells derived from central nervous system tissue from a human, fetus or adult. Again, the central nervous system tissue may be hippocampus, cerebral cortex, striatum, septum, diencephalon, mesencephalon, hindbrain, or spinal cord.

In the method for the differentiation of an *in vitro* culture of stem cells of the central nervous system of a mammal, where the stem cells maintain the multipotential capacity to differentiate into neurons, astrocytes, and oligodendrocytes, cells from the central nervous

system are:

a) dissociated by mechanical trituration;

b) plated at the optimal initial density of 1×10^6 cells (from hippocampus and septum) or 1.5×10^6 cells (from other CNS regions) per 10 cm plate precoated with poly-ornithine and fibronectin;

c) cultured in the complete absence of serum;

d) supplied daily with a growth factor selected from the group consisting of

i) basic fibroblast growth factor (bFGF) at a concentration of at least 10 ng/ml,

ii) EGF at a concentration of at least 10 ng/ml,

iii) TGF-alpha at a concentration of at least 10 ng/ml, and

iv) acidic FGF (aFGF) at a concentration of at least 10 ng/ml plus 1 μg/ml heparin;

e) replaced 100% of culture medium every two days with fresh medium;

f) passaged at every 4 days after plating by treating the cultured cells with saline solution and scraping the cells from the plate;

g) replated passaged cells at 0.5×10^6 cells per 10 cm plate precoated with poly-ornithine and fibronectin; and

h) removed the growth factor either by rinsing the cells with saline solution or by treating with trypsin and subsequently trypsin inhibitor, and then continued to culture the cells in the serum-free medium without the growth factor.

Furthermore, differentiation may be specifically directed by adding a second growth factor to the cultured cells either before or after removing the first growth factor from the cultured cells. The second or added growth factor may be platelet-derived growth factor (PDGF), ciliary neurotropic factor (CNTF), leukemia inhibitory factor (LIF), or thyroid hormone, iodothyronine (T3).

The present invention also discloses an *in vitro* culture of region-specific, terminally differentiated, mature neurons derived from cultures of mammalian multipotential CNS stem cells and an *in vitro* culture method for generation of the differentiated neurons.

In the method for *in vitro* generation of region-specific, terminally differentiated, mature neurons from cultures of mammalian multipotential CNS stem cells, multipotential CNS stem cells from a specific region are cultured in a chemically defined serum-free culture medium containing a growth factor; the medium is replaced with growth

factor-free medium; the stem cells are harvested by trypsinization; plated at a density of between 100,000 to 250,000 cells per square centimeter; and cultured in a glutamic acid-free chemically defined serum-free culture medium. The specific region of the CNS from which the multipotential stems cells are derived are selected from the group consisting of cortex, olfactory tubercle, retina, septum, lateral ganglionic eminence, medial ganglionic eminence, amygdala, hippocampus, thalamus, hypothalamus, ventral and dorsal mesencephalon, brain stem, cerebellum, and spinal cord.

In addition, the chemically defined serum-free culture medium may be selected from N2 (DMEM/F12, glucose, glutamine, sodium bicarbonate, 25 μg/ml insulin, 100 μg/ml human apotransferrin, 25 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, pH 7.2[8]) or N2-modified media. The growth factor may be selected from the group consisting of bFGF, EGF, TGF-alpha and aFGF.

Furthermore, the glutamic acid-free chemically defined serum-free culture medium may be supplemented with between 10-100 ng/ml of brain-derived neurotropic factor. The method is applicable to multipotential CNS stem cells derived from central nervous system tissue from any mammal, including rat and human.

The present invention also discloses *in vitro* cultures of region-specific, terminally differentiated, mature neurons derived from cultures of mammalian multipotential CNS stem cells from a specific region of the CNS. The specific region from which the multipotential stems cells are derived are selected from the group consisting of cortex, olfactory tubercle, retina, septum, lateral ganglionic eminence, medial ganglionic eminence, amygdala, hippocampus, thalamus, hypothalamus, ventral and dorsal mesencephalon, brain stem, cerebellum, and spinal cord. Likewise, the *in vitro* culture of region-specific differentiated neurons may be derived from any mammalian multipotential CNS stem cell, including rat and human.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the controlled differentiation of CNS stem cells at high density. Rapidly dividing nestin-positive precursor cells were labelled with BrdU during the last 24 hours of proliferation. Differentiation was then initiated by withdrawal of bFGF (day 0) and continued for up to 6 days. At indicated times, cells were fixed and stained for BrdU and neuronal antigens. Ratios of cells double-stained for BrdU and each neuronal antigen to total BrdU positive

(BrdU+) cells are shown. Up to 50% of BrdU+ cells expressed neuronal antigens and their expression was time-dependent. MAP2 positive (MAP2+), filled circle; TuJ1 positive (TuJ1+), grey diamond; neurofilament L positive (neurofilament L+), open square; neurofilament M positive (neurofilament M+), filled triangle.

Figure 1B shows proportions of MAP2+ neurons (○), GalC+ oligodendrocytes (+), and GFAP+ astrocytes (o) in differentiated clones. Clones of various sizes ranging from 39 cells to 2800 cells were differentiated for 6 days and analyzed for two cell types per clone by double immunohistochemistry. A partial list is given in Table I and immunostaining shown in Fig. 3. The number of neurons increased with increasing clone size, constituting 50% of the clone.

Figure 1C shows a comparison of the mitogenic efficacies of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Cells (initial density of 1×10^4 per plate) acutely dissociated from E16 hippocampus (HI), E14 cortex (CTX) and striatum (ST), and adult subependymal layer (Adult) were expanded with either EGF (20 ng/ml) or bFGF (10 ng/ml). Colonies arising after 10 days of expansion were stained for nestin, an intermediate filament protein characteristic for CNS precursor cells[13,14]. Relative number of

colonies averaged from at least 2 experiments for each region are shown (bFGF = 1). Twenty- to fifty-fold more nestin+ colonies per plate were present when embryonic cells were grown in bFGF (dotted bar) than in EGF (striped bar). At high densities (1×10^6 and 2.5×10^6), bFGF condition gave 10-fold higher BrdU+/nestin+ cells than EGF. Both growth factors were equally mitogenic for the adult cells. When EGF- and bFGF-expanded clones were differentiated, neurons and oligodendrocytes were found in similar quantities; however, EGF-expanded clones gave rise to significantly higher number of GFAP+ astrocytes.

Figures 2A-D show a typical clone of CNS stem cells. Cells were marked by a circle on the plate within 24 hours of plating before the first mitosis and then expanded up to 10 days (Fig. 2A). Higher magnification view of another clone before differentiation, immunostained with anti-nestin antibody, is shown in Figure 2B. Note the homogeneous radial morphology of the nestin-positive cells consistent with the nestin-positive morphology in neuroepithelium *in vivo*. Figure 2C shows a sister clone at low magnification, which has been differentiated for 6 days and immunostained with a neuron-specific antibody, TuJ1. Note the widespread and non-localized presence of TuJ1-positive neurons

across the entire clone. A higher magnification view of the same cells is shown in Figure 2D. The TuJ1-positive cells assume typical neuronal morphology. Heterogeneous morphologies in the non-neuronal TuJ1-negative cells are apparent.

Figures 3A-J show examples of representative clones of embryonic hippocampal cells (3A, 3C, 3E, 3G, 3I) and adult subependymal cells (3B, 3D, 3F, 3H, 3J) double-stained with combinations of antibodies to reveal different cell types within individual clones: anti-MAP2, neuronal; anti-GalC, oligodendrocytic; anti-GFAP, astrocytic. The two immunoreactions were developed sequentially and distinguished by using two distinct chromogens via alkaline phosphatase reaction (blue, indicated by arrows) versus horse radish peroxidase reaction (red, indicated by arrow heads).

The cells in Figures 3A and 3B were double-stained with anti-MAP2 (neuronal, arrows) and anti-GFAP (astrocytic, arrow heads) and show that bFGF-expanded clones derived from embryonic or adult brain differentiate into both neurons and astrocytes. (Oligodendrocytes are unstained in this staining.)

The cells in Figures 3C and 3D were double-stained with anti-GalC (oligodendrocytic, arrows) and anti-GFAP (astrocytic, arrow heads)

and show that bFGF-expanded clones derived from embryonic or adult brain differentiate into both oligodendrocytes and astrocytes. (Neurons are unstained in this staining.)

Figures 3E and 3F show clones differentiated in the presence of platelet-derived growth factor (PDGF). The cells were double stained with anti-MAP2 (neuronal, arrows) and anti-GFAP (astrocytic). Most cells were MAP2+ and only a few were GFAP+.

Figures 3G and 3H show clones differentiated in the presence of ciliary neurotrophic factor (CNTF). The cells were double-stained with anti-MAP2 (neuronal) and anti-GFAP (astrocytic, arrow heads). All cells were intensely GFAP+.

Figures 3I and 3J show clones differentiated in the presence of thyroid hormone, tri-iodothyronine (T3). The cells were double-stained with anti-GalC (oligodendrocytic, arrows) and anti-GFAP (astrocytic, arrow heads). GFAP+ and, particularly, GalC+ cells increased. MAP2+ cells decreased (Table IV).

Figure 4 shows the differentiation of human CNS stem cells into neuron in high density culture. bFGF-expanded CNS cells at high density were differentiated by withdrawal of bFGF ("WD"). The number of neurons expressing tau protein was determined by immunocytochemistry in culture

during the expansion phase ("Before WD") versus after differentiation ("After WD"). The dramatic increase in postmitotic neurons only after the withdrawal of bFGF indicates that they were generated from the dividing stem cells.

Figures 5A-F show human stem cells stained with human-specific anti-tau antiserum (Chemicon) which identify neurons. Proliferating human CNS stem cells in high density culture do not express tau protein, a neuronal marker (Figure 5A). After 6 days of differentiation, however, many cells with typical neuronal morphology express high level of tau protein (Figure 5B). In order to further demonstrate that these neurons have indeed derived from dividing stem cells, the stem cells were labeled with 10 μM bromodeoxyuridine (BrdU), an indicator of mitosis, for 24 hours just prior to the bFGF withdrawal. They were then differentiated for 6 days, double stained with human specific anti-tau antiserum (FITC, green) and anti-BrdU antibody (Rhodamine, red). Figure 5C shows a high magnification view of subsequent tau-positive neurons as seen through FITC fluorescence. Figure 5D shows the same field of view as in Figure 5D but seen through rhodamine fluorescence to reveal BrdU-positive nuclei. Most tau-positive neurons are also positive for BrdU, demonstrating that they were derived from mitotic

stem cells before the bFGF withdrawal.

In order to further demonstrate multi-potentiality of human CNS stem cells, they were cultured at clonal density as described for rodent cells. Figure 5E shows a typical clone at low magnification, which has been expanded from a single cell for 20 days, subsequently differentiated for 12 days, and immunostained with the neuron specific, anti-MAP2 antibody. Neurons are abundant in the clone. Figure 5F shows a higher magnification view of the clone in Figure 5E to indicate that the MAP2-positive cell are of typical neuronal morphology.

Figures 6A-D demonstrate directed differentiation of human CNS stem cells. Human CNS stem cells after 16 days of expansion were grown clonally for an additional 20 days and then differentiated in the presence or absence of single factors, PDGF (10 ng/ml), CNTF (10 ng/ml), or T3 (3 ng/ml). Figure 6A shows a untreated control clone, with approximately 50% MAP2-positive neurons (arrows) and 2-10% GFAP-positive astrocytes (arrow heads). Figure 6B shows a PDGF-treated clone, where 75% of cells are MAP2-positive neurons (arrows) and 2-10% GFAP-positive astrocytes (arrow heads). Figure 6C shows a CNTF-treated clone, where 85% are GFAP-positive astrocytes (arrow heads) and only 9%

MAP2-positive neurons (arrows). Figure 6D shows a T3-treated clone with increased number of O4- and/or GalC-positive oligodendrocytes (arrows) and of GFAP -positive astrocytes (arrow heads).

Figures 7A-7I show that a large number of mature neurons with correct axon-dendritic polarity and synaptic activity can be obtained routinely from long-term expanded CNS stem cells. Hippocampal stem cells from E16 rat embryos were expanded in culture for 16 days and through 4 passages. Just before the last passage, rapidly dividing cells were labeled with 10 μM BrdU for overnight, passaged by using trypsin, and plated onto chamber slides. Cells were maintained for 21 days to allow constitutive differentiation and maturation of neurons. Subsequently, the extent of maturation and neuronal subtypes generated were analyzed by immunocytochemistry.

Fig. 7A: Neurons stained with TuJ1 antibody viewed at low magnification (100x) to illustrate that production of neurons is efficient.

Fig. 7B: Typical morphology of neurons revealed by TuJ1 antibodies (400x).

Fig. 7C: Typical morphology of neurons revealed by MAP2 antibodies (400x).

Fig. 7D: Neurons stained with synapsin antibody. Only mature neurons containing synaptic vesicles are stained.

Fig. 7E: BrdU staining. All cells in the culture, neurons and glia, are labeled with BrdU.

Fig. 7F: Synapsin and BrdU double staining. Mature synapsin-positive cells are also BrdU-positive, demonstrating that they are derived from mitotic stem cells in culture.

Fig. 7G: Punctate anti-synapsin antibody staining marks the presynaptic axon terminals specifically in large mature neurons.

Fig. 7H: The synapsin-positive structures are closely apposed to dendritic processes revealed by MAP2 antibody staining.

Fig. 7I: MAP2 and synapsin proteins are closely associated but not co-localized, suggesting presynaptic-postsynaptic interaction.

Figure 8 shows that stem cell-derived neurons express various neurotransmitter receptors and transporters expected to be involved in synaptic transmission as detected by RT-PCR. Long-term expanded stem cells derived from E16 rodent cortex were differentiated for 14 days and harvested to prepare RNA. Undifferentiated stem cells were also prepared in order to compare differentiation-specific induction. RNA from a whole brain of adult rat was used as a positive control. Primers specific for NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3- hydroxy-5-methyl-4-isoxazole proprionic acid) families of glutamate receptor

subtypes as well as for various GABA transporters were used. Note especially the specific induction of NMDA R1, AMPA R1 and AMPA R2 receptors in differentiated cells.

Figures 9A-F show examples of typical neurons derived from rat embryonic hippocampal stem cells which had been expanded *in vitro* for 16 days (approximately 16 cell divisions through 4 passages) and differentiated for 21 days total. Mitotic CNS stem cells were pulse-labeled with bromodeoxyuridine (BrdU) for the last 24 hours prior to differentiation. Resulting neurons were triple-immunostained with antibodies against BrdU (Fig. 9A), MAP2ab (Fig. 9B), and synapsin (Fig. 9C). The composite view of the triple stained cell is shown in Fig. 9D. The BrdU-labeling demonstrates that the differentiated neuron derived from a mitotic precursor in the culture and that it is a terminally differentiated neuron since it retained the mitotic label during the prolonged differentiation phase. MAP2ab is a well-established neuron-specific protein present only in mature neurons and localized mostly in dendrites. Synapsin is a well-established synaptic vesicle protein and thus localizes synaptic terminals in axons. The triple-labeled neurons as shown in Fig. 9D established that long-term expanded mitotic CNS stem cells terminally

differentiate into mature neurons with proper subcellular polarization containing distinct dendritic (post-synaptic) and axonal (presynaptic) structures expected of fully functional neurons. Other synaptic vesicle proteins also localize in the same pattern of punctate axon terminals apposed to soma and dendrites. Fig. 9E and Fig. 9F show another hippocampal CNS stem cell derived mature neuron double-stained for synaptophysin and MAP2ab, respectively.

Figure 10 shows a field of neurons from hippocampal CNS stem cells viewed by transmission electron microscopy. The abundant presence of synapses containing synaptic vesicles and post-synaptic densities are evident.

Figures 11 A-D show intracellular electrophysiological recordings from single neurons obtained from rat E15.5 septal CNS stem cells. Consistent with the morphology, these recordings show that the CNS stem cell-derived neuronal networks are also electrophysiologically active. Thus, when individual cells were stimulated with electrode, they conducted action potentials (Fig. 11A), demonstrated presence of various voltage-sensitive ion channels (Fig. 11B), and evoked excitatory and inhibitory postsynaptic potentials in response to bath application of the excitatory neurotransmitter, glutamate (Fig. 11 C

and D). These examples establish beyond doubt that CNS stem cells give rise to terminally differentiated, electrophysiologically functional, neuronal networks.

Diverse neuronal phenotypes seen *in vivo* are obtained from the CNS stem cell cultures. Examples of some of these neuronal phenotypes are shown in Figures 12-18 and Table VII.

Figure 12 shows the expression of dopamine receptors D1 and D2 from CNS stem cells isolated from E15.5 lateral and medial ganglionic eminence. Total RNAs were isolated from respective CNS stem cell cultures differentiated for varying periods (0-20 days). Shown is the electrophoresis pattern of the DNA amplified by RT-PCR (reverse transcription-polymerase chain reaction). Results from five independent culture preparations, run in parallel in a single gel, are shown. The numbers above the lanes indicate the days of differentiation. D1 = dopamine receptor, D1; D2 = dopamine receptor, D2.

Figures 13 A-D show cholinergic neurons from septal CNS stem cells. CNS stem cells derived from E16 septum were differentiated for 18-21 days. The cholinergic neurons were assessed by acetylcholine esterase histochemistry (not shown), by immunostaining for acetylcholine transferase (Fig. 13A) and for acetylcholine transporter (Fig.

13C). In each case, CNS stem cells were incubated with the mitotic label, BrdU (10 μM), for 24 hours just before switching to the differentiation condition (Fig. 13B and D).

Figures 14A-F show neuropeptide-containing neurons obtained from rat 15.5 lateral ganglionic eminence (striatum) CNS stem cells. They are a neuropeptide Y-positive (Fig. 14A), BrdU-positive (Fig. 14B) neuron, a met-enkephalin-positive (Fig. 14C), BrdU-positive (Fig. 14D) neuron, and a leu-enkephalin-positive (Fig. 14E), BrdU-positive (Fig. 14F) neuron.

Figures 15A-F show typical morphologies of several subtypes of neurons derived from CNS stem cell of rat E12.5 ventral mesencephalon. Figure 15A and B show a TH-positive and BrdU-positive neuron (Fig. 15A-TH staining; Fig. 15B-BrdU staining). Figure 15 C and D show another TH-positive and MAP2ab-positive neuron (Fig. 15C-TH staining; Fig. 15D-MAP2ab staining). Figure 15E shows neurons stained with anti-GABA antibody. Figure 15F shows neurons stained by acetylcholine esterase histochemistry.

Figures 16A-D show examples of neurons from spinal cord stem cells. Figure 16A shows an acetylcholine esterase-positive neuron derived from rat E13.5 spinal cord CNS stem cells, which is also BrdU-positive (Fig. 16B). Cholinergic

neurons are shown by acetylcholine transferase staining (Fig. 16 C), which are also BrdU-positive (Fig. 16D).

Figures 17A and B show GABAergic neurons derived from rat E15.5 hippocampal CNS stem cells, which have been double-stained for glutamic acid decarboxylase (Fig. 17A) and GABA (Fig. 17B). Figure 17C and D show a hippocampal calretinin-positive (Fig. 17C), MAP2ab-positive (Fig. 17D) neuron.

Figures 18A-F show neurons derived from rat E13.5 thalamus and hypothalamus CNS stem cells. Figure 18A shows thalamic neurons stained for tau; Figure 18B shows the same field of view stained for BrdU. Figure 18C shows a hypothalamic neuron stained for tau; Figure 18D shows the same field of view stained for BrdU. Figure 18E and F show synapsin-positive neurons from thalamus and hypothalamus CNS stem cells, respectively.

DETAILED DESCRIPTION OF THE INVENTION

In this application, conditions are defined which permit mass expansion up to 10^9 fold in culture and controlled differentiation of multipotential CNS stem cells from the embryonic and adult brain of mammals. In both cases, clones derived from single cells differentiate into neurons, astrocytes, and oligodendrocytes.

Addition of single factors can dramatically shift the proportion of cell types within a clone.

The procedure for isolating, propagating, and differentiating the CNS stem cells are given in detail below. The procedure contains four essential steps that must be followed in concert for successful isolation and differentiation of the CNS stem cells. The four essential steps are as follows:

(1) The initial dissociation of cells from tissue is done by mechanical trituration and not by enzymatic digestion. With adult tissue, it is necessary to first enzymatically digest the tissue and then dissociate the cells from the tissue by mechanical trituration.

Trituration means gentle agitation of cell aggregates caused by fluid movement occurring during repetitive pipetting action by which individual cells become loose and dissociated from neighboring cells. Trituration is done in a saline solution free of divalent cations whose absence aids break-up of interactions among cell-adhesion proteins on cell surface. Rapidly dividing stem cells in the ventricular zone are only weakly adherent and simply removing the divalent cations from the medium and gentle agitation by pipetting are sufficient to dissociate the tissue into mostly single cells.

The cells are then cultured in the complete absence of serum. Even a brief exposure to serum deleteriously affects the differentiation capacity of the stem cells so that they are no longer able to differentiate into neurons and oligodendrocytes. Precoating the plates with poly-L-ornithine and fibronectin facilitates the adhesion of the cells to the plates.

(2). The CNS stem cells display an innate property to differentiate spontaneously, which reflects a regulatory mechanism controlling cell cycle depending upon the free concentration of growth factor, the mitogen. In order to suppress the differentiation of the stem cells into other cell types and to maintain homogeneity, the growth factor must be supplied daily at a concentration of 10 ng/ml or higher. The growth factor can be selected from (1) basic fibroblast growth factor (bFGF), (2) EGF, (3) TGF-alpha, or (4) acidic FGF (aFGF). If acidic fibroblast growth factor is selected, heparin at a concentration of 1 μg/ml must also be supplied.

(3) Even a continuous supply of bFGF or other selected growth factor is insufficient to inhibit differentiation if the culture is allowed to reach a critical density of greater than approximately 50%. This is most likely because of yet undefined endogenous factor(s) secreted by the

dividing cells themselves which antagonize the action of bFGF. Thus, in order to remove such factors from the culture and to reduce cell-cell interaction as much as possible, the cells must be passaged frequently at every 4 days after plating, and replating should be done at low density of approximately 0.5 x 10^6 per 10 cm plate, i.e., in the range of 1 x 10^2 to 1 x 10^6 cells per 10 cm plate, precoated with poly-ornithine and fibronectin.

(4) Passaging the cells by trypsin results in proteolytic removal of a bFGF receptor component and disables the mitogenic effect of bFGF. The turn-over rate of the receptor is sufficiently slow during which period the cells fail to recognize the mitogen and activate the differentiation pathway.

In order to circumvent this process, the cells are treated with Hank's buffered saline solution (HBSS) to remove divalent cations in the culture which disrupts the ionic interactions between the cadherins and the integrins on the cell surface and extracellular matrix proteins on the culture plate, causing the cells to round up. At this point the stem cells can be scraped from the plate with a scraper without damaging the cells.

Other cells in culture maintain tightly bound

to the plate and scraping eliminates them, thus allowing effective selection of rapidly dividing undifferentiated stem cells.

Differentiation of the CNS stem cells is achieved by simply removing the mitogen, bFGF or other selected growth factor, from the medium. Specification of the cell types, i.e., neurons, oligodendrocytes, and astrocytes, occurs constitutively. In order for the effective controlled differentiation, the cells must be in a homogeneous state which can be achieved by following steps 1-4, above.

These procedures yield a culture system for obtaining a homogeneous population of the CNS stem cells that can be differentiated into neurons, oligodendrocytes, and astrocytes with control and efficiency. The highlights of the features of this system are:

(1) production of a large number of the CNS stem cells with the potential to form many different neuronal subtypes, oligodendrocytes, and astrocytes that can be transplanted into a brain;

(2) controlled differentiation *in vitro* under serum-free conditions which allows the search for novel growth factors and cytokines;

(3) rapidly dividing cells accessible to genetic manipulation for introduction of foreign genes;

(4) generation of mature neurons *in vitro* suitable for genetic and pharmacological screening; and

(5) direct derivation of intermediate precursor cells from the stem cells for enrichment of a single population of cells.

The isolation of the CNS stem cells in the above-described manner further permits directed differentiation of the cells by treating them with specific growth factors. One practical significance of this directed differentiation to biotechnology is that a single cell type can be enriched *in vitro*. Thus, a novel application of previously discovered growth factors PDGF[37] (platelet-derived growth factor), CNTF (ciliary neurotrophic factor), and T3 (thyroid hormone, tri-iodothyronine) would be to direct the CNS stem cells to generate neurons, astrocytes, and oligodendrocytes, respectively.

Another practical significance, especially for PDGF, is that PDGF-induced neurons appear to be actually neuronal progenitors that can further proliferate and expand in culture by PDGF. These cells differentiate only to neurons or to neurons and oligodendrocytes and differ from the stem cells. Isolation of neuronal progenitors from mammalian CNS by PDGF has not been described previously.

EXAMPLES

1. Isolation of CNS Stem Cells from Embryonic Rat Brain

Rat embryonic hippocampus (gestation day 16; day of conception is day 1, Taconic Farm) were dissected in Hank's buffered saline solution (HBSS) and dissociated by brief mechanical trituration in HBSS. The cells were collected by centrifugation and resuspended in a serum-free medium containing DMEM/F12, glucose, glutamine, sodium bicarbonate, 25 μg/ml insulin, 100 μg/ml human apotransferrin, 25 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, pH 7.2[8], plus 10 ng/ml recombinant human basic fibroblast growth factor[12] (bFGF; R&D Inc.).

1 x 10^6 cells were plated per 10 cm plastic tissue culture plate precoated with 15 μg/ml poly-L-ornithine and 1 μg/ml bovine plasma fibronectin (Gibco). bFGF was added daily and media change was every 2 days. Cells were passaged at 50% confluence (4 days after initial plating) by briefly incubating them in HBSS and scraping with a cell scraper.

Cells with multipotential capacity were found throughout the developing neuroepithelium. Under identical culture conditions, similar cells could be prepared from other regions of the developing CNS including cerebral cortex, striatum, septum,

diencephalon, mesencephalon, hindbrain, and spinal cord. From E14 cortex and striatum and E16 hippocampus, approximately 70% of acutely dissociated cells responded to bFGF within 2 days of plating by undergoing mitosis.

2. Propagation of CNS Stem Cells from Embryonic Rat Brain

a) Mass Expansion

Hippocampal cells isolated from embryonic rat brains were expanded by daily addition of basic fibroblast growth factor (bFGF) in serum-free medium. Continuous supply of bFGF was important to repress differentiation and to maintain a homogeneous population of rapidly dividing cells expressing nestin, an intermediate filament protein characteristic for CNS precursor cells[13,14]. Less than 1% of the cells expressed the astroglial marker GFAP or the oligodendroglial markers, O4 and GalC.

The cells were passaged 4 days after plating during which time cell number increased rapidly with an average cell doubling time of approximately 24 hours. Passaged cells were replated at 0.5 x 10^6 cells per 10 cm plate and were allowed to propagate further. Cells could be passaged up to five times in this manner for a total of 20 days *in vitro* during which time a yield of 2^{20} cells could be ideally expected.

After this time period, the mitotic rate of the cells declined rapidly and the cells gradually lost their multipotential capacity, exhibiting glial characteristics and unable to differentiate into neurons.

Large numbers of cells from cortex, striatum, and septum isolated from embryos of 12-18 days of gestation could also be expanded in mass culture in the same manner. The time course of the expansion was similar to that of hippocampal cells. Continuous expansion was again limited by the constitutive loss of multipotentiality after about 20 days of cell division. Thus, this regression appears to be a characteristic property of CNS stem cells.

b) Clonal expansion

No simple antigenic marker is available which uniquely identifies multipotential stem cells from other precursors *in vitro*. Identity of a precursor population can only be ascertained by the cell's differentiation capacity. The conditions defined for mass culture in this application also permitted clonal expansion where cells were plated at extremely low cell density so that single cells were well isolated.

Differentiation capacity of the cells expanded in mass culture was assessed at each passage by plating 200 cells per 10 cm plate and

cultured under conditions as described above. Within 24 hours of plating, well isolated single cells were marked with a 3 mm ring (Nikon) on the bottom of the plate. Initial viability of the marked single cells was 5-10% and each plate typically yielded 10-20 marked clones. Only a single cell resided in each circle. The subsequent population of cells within each circle are progeny of that single cell. Clones were expanded for up to 10 days (500-2000 cells). Average double time was approximately 24 hours.

3. Differentiation and Analysis of CNS Stem Cells from Embryonic Rat Brain

Developmental potential of expanded cells was tested by directly differentiating the cells. Withdrawal of bFGF initiated differentiation within 24 hours. To initiate differentiation of high density cells, rapidly dividing cells, which had been in culture for 12 days and passaged three times, were incubated for the last 24 hours with 10 μM BrdU (bromodeoxyuridine) prior to passaging. 80-85% of the cells incorporated BrdU. The cells were harvested either by scraping or by using trypsin followed by soybean trypsin inhibitor in the serum-free medium. They were plated in duplicate at 40,000 cells/cm^2 into multi-well chamber slides (LabTek) precoated with poly-L-ornithine and fibronectin, and cultured in

the serum-free medium without bFGF. At indicated times, the cells were fixed and stained with various antibodies according to standard procedure.

Immunopositive cells were counted under 400x magnification. At least five fields with a total cell number greater than 1,000 per sample were counted. Results shown (Fig. 1A) are cell counts averaged from two experiments. Antibody reagents used were: anti-nestin antiserum; monoclonal anti-MAP2 (clone HM-2, Sigma) and anti-tau antiserum (Sigma), monoclonal anti-neurofilament L and M (clones NR4 and NN18, Boehringer-Manheim), anti-beta tubulin type III (TuJ1), monoclonal anti-GFAP (ICN), A2B5 (ATCC), O4, and anti-galactocerebroside (GalC).

Over a 6 day period, there was a progressive increase in the number of cells expressing several well established neuron-specific antigens, including MAP2a, b and c, beta tubulin type 3 (TuJ1), tau, and neurofilaments L, M, and H (Fig. 1A). Up to 50% of the cells expressed the neuronal antigens and exhibited complex neuronal morphology. The remaining cells expressed GFAP, GalC/O4, or nestin. While neurons and glia have been observed previously in expanded culture, these examples are the first to establish that the differentiation of proliferating precursor cells

can be initiated at a precise time point and that multiple cell types arise rapidly. These conditions permit large scale lineage analysis *in vitro*.

To determine if the precursor population contains separate committed progenitors that independently give rise to neurons and glia, rapidly dividing cells were plated at clonal density (200 cells per 10 cm plate) and well-isolated single cells were marked with 3 mm diameter circles. 5-10% of the marked single cells survived and proliferated with a doubling time of 24 hours to generate clones. After various periods of expansion (clone sizes ranging from 2^4 to 2^{10} cells), differentiation of clones was initiated by washing the plates once with HBSS and culturing in the same medium but in the absence of bFGF (Fig. 2).

For subcloning (data shown in Table II), the clonal plates were washed and briefly left in HBSS until the cells rounded up. Clones of 500-2000 cells were picked in 50 μl volume with an adjustable pipetter while viewing through a microscope. Each clone was replated in a 10 cm plate and single cells were marked and cultured as before.

Cell types within clones were analyzed during the first six days of differentiation by

double-staining with combinations of cell-type specific antibodies that react with mutually exclusive cells:

neurons = MAP2+, tau+, TuJ1+, neurofilament L+, or neurofilament M+;

astrocytes = GFAP+; and

oligodendrocytes = O4+ or GalC+.

Double staining was done sequentially using a commercial kit (Zymed) according to the manufacturer instructions. For oligodendrocyte staining, cells fixed with 4% paraformaldehyde were stained first for the cell-surface antigens O4 or GalC without permeabilization. The first antibody was developed with alkaline phosphatase reaction (blue) and the second with peroxidase reaction (red) (Zymed).

As in high density culture, by six days, 50% of the cells in a clone expressed neuronal antigens including MAP2, tau, and beta tubulin type III (Table I, Fig. 3A and C). In Table I, clones of hippocampal precursor cells were expanded, differentiated, and analyzed as described above. A partial list of typical clones are presented above. Total number of cells (clone size) and cells stained positive for cell-type specific antigens are shown. Their relative proportion is given in percentage in parenthesis. A total of 48 clones was quantified from 4

different passages in 6 separate experiments. Total Average indicates the average composition of each cell types in the 48 clones.

Neurofilament expression was delayed under these conditions. On average, 8% of the cells in a clone were GalC+ and had typical oligodendrocyte morphology. An additional 8% expressed GFAP and displayed a characteristic astrocytic morphology. The remaining cells were unstained by any of the antibodies specific for differentiated cell types but reacted with A2B5 and/or anti-nestin antibodies. A maximum of 20% of the cells died during differentiation. Identical results were obtained whether clones were obtained from acutely dissociated cells with no prior passage or from cells after 4 passages (26 days *in vitro*).

Cells with multipotential capacity were found throughout the developing neuroepithelium. Under identical culture conditions, similar cells could be prepared from other regions of the developing CNS including cerebral cortex, striatum, septum, diencephalon, mesencephalon, hindbrain, and spinal cord. When clonally expanded, almost all of the clones contained multiple cell types defined by both morphology and antigen expression with neurons constituting 50% of the clone.

Proliferating clones of the multipotential cells contained uniform morphology and patterns of

antigen expression. Yet, the separation of neuronal and non-neuronal morphologies occurred rapidly within 24 hours and only after the mitogen withdrawal. The early neurons were evenly distributed throughout the clone without obvious polarity or localization, suggesting the absence of committed neuronal progenitors during clonal expansion. Moreover, the number of neurons increased linearly with increasing clone size and reproducibly constituted 50% of the clone (Fig. 1B).

In order to further determine if expanding clones consisted of proliferating committed progenitors, clones were picked and replated again. 10-15% of the cells gave rise to second generation clones. Again, all of the subclones contained neurons, astrocytes, oligodendrocytes, and unstained cells (Table II). More specifically, cell type composition of subclones obtained from three independent clones, HI6, HI8, HI19, are shown in Table II. A total of 84 subclones was quantified from 13 independent parental clones in two separate experiments. Only a partial list is presented. Total Average indicates the average composition of each cell type from the 84 subclones. No subclone consisted of only one cell type. These data indicate that the multipotential precursors undergo symmetric

divisions to generate daughter cells with multipotential capacity.

4. Isolation, Propagation, Differentiation, and Analysis of CNS Stem Cells from Adult Rat Brain

The subependymal layer of adult rat brain contains mitotic nestin positive cells that could be expanded in aggregate culture in the presence of epidermal growth factor (EGF) but not bFGF[15]. Some of the cells in aggregates showed neuronal and astrocytic properties. To more fully define their developmental capacity, the mitotic population (1% of 1 x 10^5 cells/brain) lining the lateral ventricle of adult rat striatum was expanded in the presence of bFGF and compared to the embryonic precursors. Forebrain slices from 250 g adult rat brains (10-20 per experiment) were prepared and the subependymal region of striatum lining the lateral ventricles were cut out under microscope in oxygenated HBSS. The cells were dissociated by incubating minced tissues at room temperature for 10 minutes with trypsin (1 mg/ml), hyaluronidase (0.7 mg/ml), and kynurenic acid (0.2 mg/ml) in oxygenated HBSS. They were washed once in HBSS with 0.7 mg/ml ovomucoid and 0.2 mg/ml kynurenic acid, resuspended, and mechanically triturated in the same solution. Dissociated cells were recovered by centrifugation and

cultured in the serum-free medium plus bFGF (10 ng/ml) as described for the embryonic cells.

The morphology and growth characteristics of the nestin-positive adult cells were similar to those of embryonic cells. Following bFGF withdrawal, marked clones differentiated into multiple cell types expressing MAP2, TuJ1, GFAP, and GalC (Fig. 3B and D). Strikingly, the same high proportion of neurons were found in differentiated clones of adult cells as in the embryonic clones (Table III). More specifically, Table III shows the cell type composition of differentiated clones derived from adult subependymal cells. 23 clones from three independent experiments were quantified.

5. Isolation, Propagation, Differentiation, and Analysis of CNS Stem Cells from Embryonic and Adult Rat Brains

Acutely dissociated cells from various regions of embryonic brain were cultured in the presence of either EGF (20 ng/ml) or bFGF (10 ng/ml) under identical conditions as described above. Acutely dissociated adult cells were prepared as described above and cultured under identical condition as the embryonic cells. The possible effect of initial cell density on the mitogenic response was tested by varying the initial cell density from 1×10^4 to 2.5×10^6 per

plate. At low density, efficiency of colony formation was measured; at high density, BrdU+/nestin+ mitotic cells per field were counted. EGF- and bFGF-expanded colonies were also differentiated by withdrawing the mitogens and cell types analyzed as described above.

Under the culture conditions of these examples, EGF was an equally effective mitogen as bFGF for adult cells (Fig. 1C) and, when clones were differentiated, they gave rise to all three cell types. EGF-expanded embryonic clones, with and without passage, also differentiated into all three cell types. Unlike the adult cells, however, EGF was at least 10-fold less effective than bFGF as a mitogen for the embryonic cells from several different regions, regardless of initial cell density (Fig. 1C). Thus, with the exception of the proliferative effects of EGF, these data reveal that the multipotential cells from embryonic and adult CNS are remarkably similar. TGFα (10 ng/ml) was also a mitogen for the multipotential cells and was indistinguishable from EGF, while aFGF (10 ng/ml) in the presence of heparin (1 μg/ml) mimicked the effects of bFGF.

6. Directed Differentiation of CNS Stem Cells from Embryonic and Adult Rat Brain

The clonal analysis suggests that the multipotential precursors are not committed prior

to mitogen withdrawal and thus extracellular signals may regulate cell type determination. We tested whether the proportion of the cell types generated within a clone could be influenced by growth factors and cytokines either during proliferation or differentiation.

Influence of growth factors on cell type specification was tested by adding them to the culture two days before the withdrawal of bFGF and during the 6 days of differentiation. Factors were added daily and medium was changed every 2 days. At the end of the 6 days, the clones were analyzed for cell type composition by double-staining as described above. Final concentrations of the factors were 10 ng/ml PDGF-AA, -AB, or -BB, 10 ng/ml CNTF, and 3 ng/ml T3.

In embryonic clones, the proportion of neurons increased significantly in the presence of PDGF (10 ng/ml, -AA, -AB, or -BB) during the differentiation. Up to 80% of the cells were neuronal with MAP2, tau, TuJ1, or NF-M expression, and fewer cells expressed O4, GalC, and GFAP (Fig. 3E and F, Table IV). More specifically, Table IV shows the average clonal composition of each cell type obtained when clones were differentiated for 6 days either in the absence (Untreated) or presence of different factors. Clonal plates were

prepared from cells after 0-3 passages. Clone size ranged from 17 to 5336 cells. Differentiated cell types were analyzed as described above.

The cells expressing the neuronal antigens showed a less mature morphology under these conditions. When treated with ciliary neurotrophic factor (CNTF), clones gave rise almost exclusively to astrocytes (Fig. 3G and H, Table IV). Remarkably, less than 1% of the cells were MAP2-positive in this condition. The CNTF-treated cells were intensely GFAP-positive and all showed a flat, astrocytic morphology. LIF showed identical effects as CNTF.

Thyroid hormone, tri-iodothyronine (T3), influenced the differentiation of the multipotential precursors toward a mixed glial fate (Fig. 3I and J, Table IV). Astrocytes and oligodendrocytes were both increased 3-fold and there was a marked decrease in the proportion of neurons. As in the untreated clones, GalC- and O4-positive cells showed characteristic oligodendrocyte morphologies. The clones were of similar size in all the experiments and numerical analysis of dead cells showed that selective cell death cannot account for the changes in the proportion of cell types. Similar results were obtained with multipotential stem cells from embryonic cortex and striatum. Furthermore, the

multipotential cells derived from subependymal layer of the adult brain showed quantitatively similar differentiation responses to PDGF, CNTF, and T3 (Fig. 3, Table IV). This emphasizes the general nature of these pathways.

Other factors that were tested during this study and showed no significant instructive effect on cell type determination were: NGF, NT-3, BDNF, TGFb1, IL1b, IL2-11, G-CSF, M-CSF, GM-CSF, oncostatin M, stem cell factor, erythropoietin, interferon gamma, 9-cis and all-trans retinoic acid, retinyl acetate, dexamethasone, and corticosterone.

7. Isolation, Expansion, Differentiation, and Analysis of CNS Stem Cells from Human Fetal Brain

Tissues from various regions of human fetal brains were obtained from fetuses of 45 to 114 days of gestation periods. The tissues were dissociated in HBSS by mechanical trituration as described above. Cells were collected by centrifugation, resuspended, plated at 1×10^6 cells per 10 cm plate, and expanded in the serum-free medium plus 10 ng/ml bFGF under conditions identical to those described for rodent fetal CNS stem cells above.

Approximately 25-50% of the human cells depending upon the age and the region had CNS stem

cell morphology and responded to bFGF by rapid cell division. Human CNS stem cells were expanded in culture for up to 36 days. Average doubling time was approximately 48 hours, which contrasts with the rodent counterpart with 24 hour doubling time. Upon withdrawal of bFGF, the differentiation of human fetal CNS stem cells occurred rapidly and multiple cell types arose. In high density culture, the cells were differentiated for up to 13 days and subsequent cell types present were analyzed by immunocytochemistry as described for rodent cell culture. Before differentiation by bFGF withdrawal, few tau-positive neurons were present in the culture (Fig. 4). In contrast, after the bFGF withdrawal, up to 40% of the bFGF-expanded human fetal brain cells in mass culture were neurons immunoreactive with human-specific anti-tau antiserum. A majority of the tau-positive neurons in culture could be labeled with BrdU (bromodeoxyuridine), the indicator of mitosis, within 24 hours prior to the bFGF withdrawal (Fig. 5C and D). This result demonstrates that the culture conditions defined for rodent CNS stem cells applies equally well for efficient expansion and differentiation of human CNS stem cells to generate large numbers of neurons in culture.

In order to further analyze the multi-

potentiality of the human fetal CNS stem cells in mass culture, dividing cells were plated at clonal density (100-200 cells per 10 cm plate) and further expanded for 20 days (clone size = 2^{10}). Subsequently, clones were differentiated by withdrawing bFGF and analyzed for cell types immunoreactive for neuron-, astrocyte-, or oligodendrocyte-specific antibodies. Almost all of clonally expanded human fetal cells differentiated to give rise to all three cell types--neurons, astrocytes, and oligdendrocytes (Fig. 5E and F). As with rodent stem cells, MAP2-positive neurons comprised approximately 50% of the clone (Table V). The remaining cells were of large elongated glial morphology. Approximately 10% of the cells expressed mature astrocytic antigen, GFAP, and about 2% expressed oligodendrocytic antigens O4 or galactocerebroside (GalC) (Table V). This clonal analysis thus demonstrates that the culture system described here permits efficient isolation, mass-expansion, and differentiation of multipotential stem cells from human fetal CNS.

8. Directed Differentiation of CNS Stem Cells from Human Fetal Brain

In addition to the mulitpotentiality and self-renewing properties of CNS stem cells, the capacity to differentiate into one cell type in

response to an extracellular signal is the key defining property of rodent CNS stem cells as demonstrated above. The three extracellular factors, PDGF, CNTF, and T3, also directed the differentiation of the human CNS cell clones in an identical manner (Table V; Fig. 6A-D). Thus, in the presence of PDGF, MAP2-positive neuronal cells increased to 71% of a clone, significantly higher than the 46% in the untreated control culture. In contrast, in the presence of CNTF, MAP2-positive cells decreased and GFAP-positive astrocytes increased dramatically to 85% of the clones. T3 increased O4- or GalC-positive oligodendroglial cells as well as GFAP-positive astroglial cells, while MAP2-positive neurons decreased (Table V). These results demonstrate the similarities quantitatively between the human and the rodent CNS stem cells and the universal applicability of the present culture system for efficient expansion and differentiation of mammalian CNS stem cells.

9. Maturation, Synaptogenesis, and Diversity of Stem Cell-Derived Neurons *In Vitro*

Multipotentiality of CNS stem cells and their directed differentiation by defined extracellular signals unequivocally establish that neurons derive from stem cells directly. Thus, the origin of neuronal diversity seen in mature brain starts from CNS stem cells. Can the CNS stem cells

expanded in culture for long-term retain the ability to mature to form axonal-dendritic polarity, to interact with other cells and form synapses? In order to investigate the extent to which CNS stem cell-derived neurons can mature *in vitro* under serum-free condition, stem cells derived from embryonic rat hippocampus were allowed to differentiate for up to 21 days at high density.

Subsequently, neurons were stained with various antibodies recognizing either axon- or dendrite- specific proteins. Synapsin, synaptophysin, synaptobrevin, and syntaxin are proteins found in synaptic vesicles of mature neurons at axon terminals and are involved in exocytosis of neurotransmitters. All four proteins were highly co-localized in the stem cell-derived neurons, in punctate pattern, most likely delineating the axon terminals. The processes bearing the synaptic vesicle proteins were thin, highly elaborate, traveled long distance, and decorated the perimeter of neighboring neurons (Fig. 7G). They contained axon-specific proteins such as tau and neurofilament and were devoid of dendrite specific proteins such as MAP2a and MAP2b (Fig. 7H and 7I).

These results indicate that, similar to neurons generated *in vivo*, the stem cell-derived

neurons display proper axon-dendrite polarity and exhibit synaptic activity. Stem cell-derived neurons also expressed major neurotransmitter receptors, transporters, and processing enzymes important for neurotransmitter functions. These included members of glutamate receptors, GABA receptors, and dopamine receptors (Fig. 8). Furthermore, the stem cells retain their capacity to generate subtypes of neurons having molecular differences among the subtypes.

10. *In Vitro* Generation of All Neuronal Subtypes Found in the Mature Brain by Differentiating CNS Stem Cells

Understanding the molecular programs that govern the organization of complex neuronal diversity in the mammalian adult brain is a major goal of developmental neurobiology. Most of the structural domains in the adult brain and subpopulations of postmitotic neurons comprising them are generated during embryonic development. The developmental properties of the immediate precursor cells that give rise to specific neurons, however, are largely unknown. Also unclear are the precise stage of differentiation and the general molecular principle by which neurons acquire their neurotransmitter phenotypes.

One emerging line of evidences is that from early stages of development, neural tube and brain

vesicles are patterned by spatial and temporal expression of a number of nuclear and secreted proteins[58,59]. This evidence is consistent with the hypothesis that the early neuroepithelium is composed of predetermined precursor cells and that mature cortical organization, for example, derives from a predetermined early "proto-cortex."[60]

The idea of predetermined neuroepithelium, however, is at odds with other observations from *in vivo* fate mapping studies and transplantation studies[5,61-63]. One main conclusion from these experiments is that certain precursor population(s) are multipotential and/or widely plastic in respect to the neuronal versus glial lineages as well as neuronal phenotypes such as neurotransmitter phenotypes and laminar or regional destination. In order to reconcile these two sets of seemingly contradicting observations, several major issues must be addressed. What is the differentiation capacity of the precursor cell that directly gives rise to terminally differentiated neurons? What information, if any, does that precursor cell contain in respect to specific phenotype of the neurons?

To answer these questions, we have successfully isolated from early rat neuroepithelium multipotential precursor cells, CNS stem cells, and examined quantitatively their

differentiation capacity *in vitro*[64] (see also Examples 1-3, 5 and 6). Under constitutive conditions with no exogenous influence, CNS stem cell clones differentiated into all three major cell types-- neurons, astrocytes, and oligodendrocytes. In the presence of single extracellular factors, however, their fate choice could be directed toward single cell types. Moreover, such multipotential stem cells were by far the majority of expandable populations in culture suggesting that they are abundant in the neuroepithelium. These properties are also shared by CNS stem cells from human fetal brain. Thus, these are the defining properties of mammalian CNS stem cells, which constitute the majority of embryonic CNS and are the direct precursors to neurons of the adult brain.

What, then, is the developmental capacity of multipotential CNS stem cells in respect to neuronal phenotypes? In this Example, we examined the extent of information embedded in the isolated CNS stem cells to guide terminal differentiation and maturation of neurons and for generation of specific subpopulations of neurons. We found that although CNS stem cells are widely distributed in large numbers throughout the neuroepithelium and are equally multipotential in respect to the three major cell types, CNS stem cells derived from a

distinct region give rise to neuronal phenotypes appropriate for that region only. We conclude that the information specifying region-specific neuronal phenotypes is present in the multipotential stem cell state, that this information is stably inherited through many cell divisions *in vitro*, and that, when differentiated under constitutive conditions in the absence of external influence, CNS stem cells are non-equivalent and each gives rise to only restricted sets of neurons appropriate for the region from where the CNS stem cells originated.

In Examples, 1-3, 5 and 6, we limited the differentiation of CNS stem cell clones only to the earliest time point of maturation at which all three cellular phenotypes, i.e., neurons, astrocytes and oligodendrocytes, could be sampled without encountering significant cell death. Hence, neuronal differentiation was limited only to early stages of differentiation. We decided to examine to what extent CNS stem cell-derived neurons could differentiate *in vitro* under constitutive conditions, that is, in serum-free, defined minimal medium in the absence of exogenous factors.

Neuronal differentiation encompasses many distinct phases of cellular maturation. One of the earliest characteristics of a functional

neuron to be expected is the polarization of a neuron into distinct compartments, i.e., soma, dendrite and axon. We examined various defined culture conditions to promote differentiation of clones of multipotential CNS stem cells into polarized neurons. Under clonal conditions, we found that neuronal survival is too limited to permit systematic characterization of late phenotypes of neuronal differentiation. Interestingly, addition of various commercially available neurotrophic factors including NGF and FGF families could not overcome this barrier.

However, simply increasing the cell density of differentiating CNS stem cells was sufficient for effective neuronal survival, and polarized neurons with mature morphology could be reproducibly obtained after 14-21 days in N2 medium in the absence of glutamate and any other exogenous factors. Although not essential, occasional supplementation of the culture with brain-derived neurotrophic factor (BDNF) further facilitated long-term neuronal survival generally and was, therefore, used in this Example.

Specifically, CNS stem cells were isolated and expanded under defined conditions as previously described above in Examples 1-3, 5 and 6. Different neurons were derived by isolating CNS stem cells from different regions of the

central nervous system and from different stages of the CNS development. Differentiation conditions for obtaining all neuronal phenotypes were identical and different neurons derived only from allowing expression of inherent information already embedded in the expanded CNS stem cells.

More specifically, at the last mitotic cycle, differentiation was overtly triggered by withdrawal of mitogen, e.g., bFGF, by replacing the growth medium with mitogen-free medium. At the same time, or some days later without any differential consequence, the cells were harvested by trypsinization and centrifugation according to conventional procedures. Trypsin was inactivated by adding trypsin inhibitor. The resulting cell pellet was resuspended in the same N2 growth medium without bFGF or any other factor and plated at high cell density, optimally at 125,000 cells per square centimeter, onto tissue culture plates precoated with poly-L-ornithine (15 μg/ml) and fibronectin (1 μg/ml) or laminin (1 μg/ml). Two to four days later, the N2 medium was replaced by N2 medium without glutamic acid. The high cell density was necessary for efficient neuronal differentiation and the absence of glutamic acid was necessary to permit long-term survival of mature neurons.

Neurons were maintained for long periods (up

to 30 days) under these conditions with the medium changed every 3-4 days. Supplementing the medium with 20 ng/ml of recombinant human BDNF further facilitated neuronal survival and maturation. After 12-30 days of differentiation, cells were fixed with 4% paraformaldehyde and neuronal phenotypes were identified by immunocytochemistry against marker proteins.

In order to directly demonstrate that the mature neurons and various subtypes of neurons in culture were directly produced from the mitotic CNS stem cells, CNS stem cells from various embryonic brain regions (see below for examples) which had been expanded *in vitro* for long-term (approximately 16 days and 16 cell divisions through 4 passages) were overtly differentiated for 21 days total as described above. Mitotic CNS stem cells were pulse-labeled with bromodeoxyuridine (BrdU) for the last 24 to 48 hours prior to differentiation. From all regions, up to 86% of MAP2ab-positive neurons were also positive for BrdU. By 24 hour-BrdU labeling, approximately 50%-75% of the neurons immunopositive for the antigens specific for different neuronal subtypes were also positive for BrdU. Prior to the overt differentiation step, no neurons expressing MAP2ab or any other subtype-specific antigens were observed in any of the CNS stem cell cultures.

Thus, consistent with the previous examples (Examples 1-8), all of the neurons and neuronal subtypes reported below were produced exclusively from long-term expanded, mitotic, CNS stem cells.

Neurons thus obtained contained distinct localization of dendritic proteins such as MAP2ab from axonal proteins such as tau, neurofilaments, and several synaptic vesicle proteins. Shown in Fig. 9 are typical neurons derived from rat embryonic hippocampal stem cells. Mature neurons were triple-immunostained with antibodies against BrdU (Fig. 9A), MAP2ab (Fig. 9B), and synapsin (Fig. 9C). Combined staining is shown in Fig. 9D.

Figures 9E and F show another typical example of hippocampal stem cell-derived neurons double-stained for synaptophysin (Fig. 9E), a synaptic vesicle protein labeling axon terminals, and MAP2ab (Fig. 9F) labeling dendritic process. These immunostaining results demonstrate polarization of neurons into axons and dendrites and significantly suggest numerous synaptic junctions. Further examination of these morphologies by electron microscopy confirmed the abundant presence of synapses containing synaptic vesicles and synaptic densities (Fig. 10).

These neuronal networks were also functional electrophysiologically. They conducted action potentials (Fig. 11A), contained various voltage-

sensitive ion channels (Fig. 11B), and transmitted excitatory and inhibitory postsynaptic potentials when evoked by bath application of the excitatory neurotransmitter, glutamate (Fig. 11C and D). Thus, these results unequivocally demonstrate that all of the information necessary and sufficient to form mature neurons and synaptogenesis from the mitotic state is self-contained and stable within the long-term expanded CNS stem cells.

Long-term expanded CNS stem cells derived from several different regions of the neuro-epithelium gave rise to distinct subpopulations of neurons. Thus, CNS stem cells were isolated from several different regions of rat embryonic CNS at times known to be at the beginning or in the midst of neurogenesis-- embryonic gestation day 15.5 (E 15.5) cortex (CTX), septum (SEP), lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), hippocampus, E13.5 thalamus, hypothalamus, E12.5 ventral and dorsal mesencephalon, and E11.5-E13.5 spinal cords. From each of these regions, almost homogeneous cultures of CNS stem cells could be expanded for long term (typically for 16 days with average doubling time of 24 hours) according to the culture conditions described previously[64].

General properties of the expanding CNS stem cells such as morphology, mitotic rate, and

differentiation characteristics were indistinguishable among different regions including hippocampus which has been described above in detail[64]. In clonal analysis, each of these regions contain many CNS stem cell clones with multipotential capacity to differentiate into neurons, astrocytes, and oligodendrocytes in relative proportions identical to the previously detailed hippocampal stem cell clones[64].

The compelling question then is whether there is only one kind of stem cell that constitutes the entire neuroepithelium and that regional specification and neuronal diversity occur at subsequent stages of development or whether the CNS stem cells at the multipotential stage contain the information for regionally distinct neuronal phenotypes.

Lateral and medial ganglionic eminence are two closely adjacent structures that develop in parallel into striatum and globus pallidus in the adult brain. Dopamine receptors, D1 and D2 are expressed in striatum, but only D2 is present in pallidus. The expression of D1 and D2 receptors from CNS stem cells isolated from E16 lateral and medial ganglionic eminence was examined by RT-PCR (Fig. 12). Prior to differentiation, CNS stem cells from either region expressed no dopamine receptors. After 9 days of differentiation, D1

and D2 receptors were expressed in LGE-derived stem cells, but only D2 receptor was expressed in cells from MGE. This differential pattern was stable throughout the differentiation course up to 21 days examined (Fig. 12).

Cholinergic neurons of septum have been critically attributed to the onset of Alzheimer's disease. These neurons appear during E15-E18 in rats. CNS stem cells derived from E16 septum were differentiated for 18-21 days under the defined conditions as described above and the presence of cholinergic neurons were assessed by acetylcholine esterase histochemistry and by immunostaining for acetylcholine transferase and for vesicular acetylcholine transporter. Figure 13A shows a septal CNS stem cell-derived cholinergic neuron immuno-stained for acetylcholine transferase. Figure 13B shows the same field of view as Fig. 13A stained for the mitotic label BrdU. Figure 13C and D show another example of a cholinergic neuron double-stained for vesicular acetylcholine transporter and BrdU, respectively.

Table VII summarizes the number of MAP2ab-positive neurons per square centimeter and the proportions of different neuronal phenotypes relative to the total MAP2ab-positive neurons derived from CNS stem cells of several different regions and ages. Approximately 4-5% of the MAP2

positive neurons were cholinergic. In contrast, hippocampal and cortical CNS stem cells gave rise to no cholinergic neurons.

About 0.4% of neurons derived from LGE and MGE CNS stem cells also expressed vesicular acetylcholine transporter, a specific marker of cholinergic neurons (Table VII). Approximately 2.8% to 10.7% of LGE and MGE-derived neurons contained several different neuropeptides such as neuropeptide Y, met-enkephalin, and leu-enkephalin (Fig. 14; Table VII). Figures 14A, C, and D show typical LGE CNS stem cell-derived neurons stained for neuropeptide Y, met-enkephalin, and leu-enkephalin, respectively. Figures 14B, D, and F show the immunostaining for BrdU of the same fields as in Figs. 14A, C, and E, respectively.

When CNS stem cells expanded from E12.5 ventral mesencephalon were differentiated, approximately 2.6 ± 0.3% of MAP2 positive neurons expressed tyrosine hydroxylase, the key enzyme for dopamine synthesis and a well-established marker of dopaminergic neurons (Table VII). Figure 15A shows a typical CNS stem cell derived TH-positive neuron and Figure 15B shows the corresponding BrdU staining of the same field. All TH-positive cells are neurons as shown by double-staining for TH and MAP2ab (Figs. 15C and D, respectively). Most of the remaining neurons were positive for the marker

of GABAergic neurons, glutamic acid decarboxylase (GAD) as well as for GABA itself (Fig. 15E) and/or for acetylcholine esterase (Fig. 15F; Table VII) which is known to be expressed in monoaminergic neurons in this area.

CNS stem cells derived from dorsal mesencephalon, in contrast, generated no TH-positive neurons (Table VII). Almost all neurons of this area (100.9 ± 9.1%) expressed acetylcholine esterase (Table VII). They are most likely monoaminergic neurons, consistent with the *in vivo* pattern. Significantly, no TH-positive neurons arose from CNS stem cells derived from cortex, septum, hippocampus, striatum, and spinal cord (Table VII). Thus, in parallel with the known *in vivo* expression pattern, generation of TH-positive neurons were unique to ventral mesencephalon CNS stem cells *in vitro*.

CNS stem cells from E13.5 cervical and thoracic spinal cords were expanded and differentiated. 1.2 ± 0.1% of MAP2 positive neurons were cholinergic containing vesicular acetylcholine transporter (Table VII). Cholinergic neurons also expressing acetylcholine transferase and BrdU-positive are shown in Figures 16C and D, respectively. 39.3 ± 2.5% of the neurons expressed acetylcholine esterase (Table VII), most of which are expected to be

monoaminergic. A typical acetylcholine esterase-positive and BrdU-positive neuron is shown in Figure 16A and B, respectively.

Neurons derived from E15.5 hippocampal and cortical CNS stem cells did not express tyrosine hydroxylase, acetylcholine esterase, acetylcholine transferase, and vesicular acetylcholine transporter (Table VII). This is appropriate for known absence of these markers in hippocampus *in vivo*. About 30% of MAP2ab-positive neurons were GABAergic, indicated by expression of GAD and GABA. Figures 17A and B show typical examples of GAD- and GABA-positive staining, respectively, which completely overlap. A typical hippocampal calretinin- and MAP2ab-positive neuron is shown in Figures 17C and D, respectively.

Mature neurons can be also be derived with equal efficiency from E13.5 thalamus and hypothalamus. These neurons contain exceptionally long axonal processes. A typical thalamic neuron stained for the axonal protein, tau, and BrdU is shown in Figures 18A and B, respectively. A typical hypothalamic neuron stained for tau and BrdU is shown in Figures 18C and D, respectively. Synapsin staining of thalamic and hypothalamic neurons is shown in Figures 18E and F, respectively.

The examples given above have been selected

based upon only well-established *in vivo* populations available in the literature and also upon the well-defined markers commercially available. A summary of the proportions of different neuronal phenotypes from various regional CNS stem cells are shown in Table VII. These examples are only a part of the neuronal diversity present in the CNS stem cell-derived cultures.

In summary, these results conclusively demonstrate that distinct subpopulations of neurons are generated in culture from expanded CNS stem cells and that the types of neurons generated are restricted in a region-specific manner approximately corresponding to *in vivo* patterns of expression. The information which specifies neuronal phenotype is therefore embedded in the multipotential stem cell state. Moreover, this specifying information is heritably stable through many cell divisions and enacted during the subsequent differentiation process. These results directly demonstrate that the mammalian neuroepithelium is indeed divided in a mosaic pattern of different kinds of multipotential CNS stem cells with heritable, restricted information which specify neuronal phenotypes in the absence of any other interactions. Thus, all neuronal subtypes found in the mature mammalian brain can

be generated *in vitro* by differentiating appropriate CNS stem cells.

These neurons and the CNS stem cells capable of differentiating into such neurons provide the key element for gene therapy, cell therapy, and identification of novel therapeutic molecules (proteins, peptides, DNA, oligonucleotides, synthetic and natural organic compounds) directed to nervous system disorders.

SIGNIFICANCE OF CNS STEM CELL TECHNOLOGY

Behavior of the stem cells *in vitro* provides important insights on the CNS development. Efficient proliferation and controlled differentiation of the precursor cells permitted a quantitative analysis of their developmental capacity. They display properties expected of stem cells: rapid proliferation, multi-potentiality, and self-regeneration. Moreover, the cells from adult brain were quantitatively equivalent to the embryonic cells, indicating that stem cells persist in the adult.

The multipotential cells could be efficiently isolated from many regions of the developing CNS, indicating that they are abundant throughout the neuroepithelium. This contrasts with the widely-held notion that stem cells are rare. Differentiation of the stem cells can be

effectively directed by extracellular factors that are known to be present during CNS development[16-23]. This suggests that different extracellular factors can act on a single class of stem cells to generate different cell types. A similar instructive mechanism has also been observed *in vitro* with stem cells isolated from the peripheral nervous system[24].

Multiple cell types appear rapidly when the stem cells are differentiated *in vitro*. In contrast, neurons, astrocytes, and oligodendrocytes appear at distinct times *in vivo*. Clearly, additional mechanisms must regulate the fate choice *in vivo*. Temporal and spatial expression of extracellular factors and their receptors may be a part of the mechanism.

Another mechanism of fate choice regulation *in vivo* may involve intermediate stages of differentiation. Identification of the bipotential oligodendrocyte precursor cell, O-2A, from postnatal optic nerve directly demonstrated that restricted progenitors are produced during development[25,26]. The stem cells are distinct from the O-2A cells. Their origins, properties, and developmental capacities differ. Given that the stem cells differentiate into oligodendrocytes, the differentiation pathway may involve an obligatory intermediate stage, a committed

progenitor state like the O-2A cell. The similar responses of both cells to T3 and CNTF[27,28] may reflect this common step.

There is also evidence from lineage analysis *in vivo* and *in vitro* for the presence of other lineage restrictions, including bipotential neuronal and oligodendrocyte precursors and committed neuronal progenitors[6,7,29,30]. These may also arise from differentiating stem cells. The clonal assay described here will permit the relative contributions of lineage commitment versus instructive and selective factors on these intermediate cells to be quantitatively defined.

In summary, the present application reveals that:

(1) most regions of the fetal brain and spinal cord can be made to multiply in culture under completely defined culture conditions to yield up to a 1,000,000,000-fold increase in cell number;

(2) the homogeneous stem cell culture can be triggered to differentiate under precisely controlled conditions where up to 50% of the cells differentiate into neurons while the remaining cells become astrocytes and oligodendrocytes;

(3) many different kinds of neurons are generated in culture;

(4) growth factors have been identified that

effectively direct the stem cells to differentiate into a single cell type, i.e., neuron, astrocyte, or oligodendrocyte; and

(5) equivalent stem cells have been isolated and expanded from adult subependymal layer by using a similar procedure.

These enumerated results provide the following advantages over the current state of the art. First, this CNS stem cell technology permits large scale culture of homogeneous stem cells in an undifferentiated state. The longer that the cells can be maintained in the stem cell state, the higher the yield of neurons that can be derived from the culture, thereby enabling more efficient gene transfer and large scale selection of those cells carrying the gene of interest.

Second, this culture system permits controlled differentiation of the stem cells where 50% of the expanded cells now turn into neurons. This efficient differentiation, combined with efficient proliferation, routinely yields more than 100 million neurons from the neocortex of one rat fetal brain in a two-week period.

Third, the differentiation of the stem cells into neurons, astrocytes, and oligodendrocytes occurs constitutively where all three cell types continue to mature in culture, most likely due to nurturing interactions with each other, as during

normal brain development. Many different types of neurons arise, which respond to many growth factors and contain neurotransmitters and their receptors. Thus, a significant portion of the brain development can be recapitulated in a manipulable environment, thereby highlighting the potential to extract and test novel neurotropic factors normally secreted by these cells.

Finally, these results permit the establishment of conditions by which dividing immature neurons can be derived directly from the stem cells and expanded further to allow large scale isolation of specific kinds of neurons in culture.

POTENTIAL COMMERCIAL APPLICATIONS

The stem cell technology of the present invention can be developed for direct application to many different aspects of therapy and drug discovery for nervous system disorders. Outlined below are four examples for potential commercial applications, i.e., gene therapy for Parkinson's disease, cell therapy, search for novel growth factors, and assays for drug screening.

The CNS stem cells more than meet the technical criteria as vehicles for gene therapies and cell therapies in general. The stem cells can be expanded rapidly under precisely controlled,

reproducible conditions. Furthermore, these cells are readily accessible to all standard gene transfer protocols such as via retroviruses, adenoviruses, liposomes, and calcium phosphate treatment, as well as subsequent selection and expansion protocols. The expanded stem cells efficiently differentiate into neurons *en masse*.

In addition, it should be emphasized that two additional properties of the stem cells make them unique as the fundamental basis of therapeutic development directed at the human nervous system. First, once stem cells are triggered to differentiate into mature cell types, all of the molecular interactions are in place within the culture system to generate, to mature, and to survive a variety of different cell types and neuronal subtypes. These interactions recapitulate a significant portion of the natural brain development process. Therefore, the stem cells, as vehicles of gene therapy and cell therapy, refurnish not only a single potential gene or factor to be delivered but also the whole infrastructure for nerve regeneration.

Second, the stem cells in culture are expanded from the multipotential germinal precursors of the normal brain development. Hence, these stem cells retain the capacity to become not only three different cell types but

also many different types of neurons depending upon the environmental cues to which they are exposed. This broad plasticity, which is the inherent property of the stem cells, distinctly suggests that, once transplanted, the cells may retain the capacity to conform to many different host brain regions and to differentiate into neurons specific for that particular host region. These intrinsic properties of the primary stem cells are far different from the existing tumorigenic cell lines where some neuronal differentiation can be induced under artificial conditions. Therefore, with these unique properties, the expandable human CNS stem cells contain significant commercial potential by themselves with little further development.

1. Gene Therapy for Parkinson's Disease

Parkinson's Disease results mainly from degeneration of dopamine releasing neurons in the substantia nigra of the brain and the resulting depletion of dopamine neurotransmitter in the striatum. The cause of this degeneration is unknown but the motor degeneration symptoms of the disease can be alleviated by peripherally administering the dopamine precursor, L-dopa, at the early onset of the disease. As the disease continues to worsen, L-dopa is no longer effective

and currently no further treatment is available. One promising treatment being developed is to transplant dopamine-rich substantia nigra neurons from fetal brain into the striatum of the brain of the patient. Results obtained from various clinical centers look extremely optimistic. However, it is estimated that up to 10 fetal brains are needed in order to obtain enough cells for one transplant operation. This requirement renders unfeasible the wide application of the transplantation of primary neurons as a therapeutic reality. This is exactly the type of problem solved by the CNS stem cell technology of the present application, whereby a small number of cells can be expanded in culture up to a 1,000,000,000 fold.

It is now widely recognized that transplantation of dopamine producing cells is the most promising therapy of severe Parkinson's Disease and that a stable cell population or cell line genetically engineered to produce dopamine is essential to effective therapy. Tyrosine hydroxylase (TH) is the key enzyme for dopamine synthesis. Human CNS stem cells derived from fetal basal ganglia can be produced which express the tyrosine hydroxylase (TH) gene. These cells can be expanded, differentiated, and transplanted into the patient's striatum. Since the cells are

originally derived from the primordial striatum, they would have the best chance of integrating into this region of the brain. Production of such cells and their successful transplantation into animal models will result in the most promising application of gene therapy to date.

2. Cell Therapy

In most neurological diseases, unlike Parkinson's Disease, the underlying cause of symptoms cannot be attributed to a single factor. This condition renders the therapeutic approach of introducing a single gene by gene therapy ineffective. Rather, replacement of the host neuronal complex by healthy cells will be required. Since CNS stem cells are the natural germinal cells of the developing brain with the capacity to become the cells of the mature brain, the stem cells from the spinal cord and different regions of the brain may be used directly to repopulate degenerated nerves in various neuropathies.

Several specific stem cell lines that over express various growth factors that are currently in clinical trials are being developed. This application combines the unique plasticity of the stem cells and growth factor-mediated gene therapy to provide not only the benefit of the targeted

delivery of the protein but also more broad neuronal regeneration in specific areas.

Primary examples of growth factors currently in clinical trials or under full development by various companies are listed below in Table VI[31]. So far, tests of growth factors have been limited to direct peripheral injection of large doses, which brings significant risks of side effects since most growth factors affect many different populations of neurons and non-neural tissues and with a short half-life. These problems can be overcome by generating from the CNS stem cells several cell populations or cell lines stably expressing these growth factors and demonstrating their capacity to differentiate into neurons and to secrete the growth factors in specific peripheral and central regions.

3. Search for Novel Growth Factors

One of the central principles of modern neurobiology is that each of the major projection neurons, if not all neurons, requires specific signals (trophic factors) to reach their target cells and survive. Neuropathies in many diseases may be caused by or involve lack of such growth factors. These growth factors represent the next generation of preventive and therapeutic drugs for nervous system disorders, and hence the enormous capitalization invested in the search and

development of novel growth factors by the biotechnology industry.

Implicit in the observation that a variety of mature neurons can be produced from CNS stem cell culture is that various growth factors are secreted by the differentiating cells for determination of cell types, maturation, and continued support for their survival and that the cells contain the necessary receptor machinery to respond to those growth factors and probably others. Most of the growth factors known so far in the nervous system were discovered by their effects on peripheral nerves and they most likely represent a very minor fraction of existing growth factors in the brain.

Search for growth factors from the brain has been difficult mainly because particular neuronal cell types are difficult to isolate from the brain and maintain in defined culture conditions. Differentiation of the stem cells into neurons overcomes this problem and opens new assays to screen potential growth factors.

4. Assays for Drug Screening

As more and more neurotransmitter receptors and signal transducing proteins are being identified from the brain, it is becoming clear that the dogma of one neurotransmitter activating

one receptor is an oversimplification. Most receptor complexes in neurons are composed of protein subunits encoded by several genes and each gene synthesizes many different variations of the protein. These variations result in a wide range of possible receptor combinations, and not a single receptor, that can interact with a neurotransmitter. Consequently, a range of signal output may be produced by a single neurotransmitter action. The specific signal effected by a neurotransmitter on a neuron, then, depends on which receptor complex is produced by the cell. Thus, cellular diversity must parallel the molecular diversity and constitute a major structural element underlying the complexity of brain function.

Drug discovery by traditional pharmacology had been performed without the knowledge of such complexity using whole brain homogenate and animals, and mostly produced analogs of neurotransmitters with broad actions and side effects. The next generation of pharmaceutical drugs aimed to modify specific brain functions may be obtained by screening potential chemicals against neurons displaying a specific profile of neurotransmitters, receptors complexes, and ion channels.

CNS stem cells expanded and differentiated

into neurons in culture express several neurotransmitters and receptor complexes. Many cell lines derived from stem cells and neuronal progenitors of different regions of the brain can be developed which, when differentiated into mature neurons, would display a unique profile of neurotransmitter receptor complexes. Such neuronal cell lines will be valuable tools for designing and screening potential drugs.

In summary, the CNS stem cell technology of this application offers broad and significant potentials for treating nervous system disorders.

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While the invention has been described in connection with what is presently considered to be the most practical and preferred embodiments, it is to be understood that the invention is not limited to the disclosed embodiments, but on the contrary is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus, it is to be understood that variations in the present invention can be made without departing from the novel aspects of this invention as defined in the claims. All patents and scientific articles cited herein are hereby incorporated by reference in their entirety and relied upon.

TABLE I:
CELL TYPE COMPOSITION OF DIFFERENTIATED CLONES
Clones of Embryonic Hippocampal Precursor Cells

Passage	Clone Size	MAP2+ (%)	GalC+ (%)	GFAP+ (%)
1	319	145 (45)		41 (13)
1	451	245 (54)		0 (0)
1	1237	634 (51)		9 (1)
1	2197	956 (44)		42 (2)
1	2779	1617 (58)		336 (12)
4	71		10 (14)	5 (7)
4	139		14 (10)	4 (3)
4	296		21 (7)	139 (47)
4	341		54 (16)	38 (11)
4	420		39 (9)	25 (6)
4	600		35 (6)	60 (10)
4	662		66 (10)	62 (9)
4	141	42 (30)	4 (3)	
4	427	220 (52)	15 (4)	
4	610	306 (50)	29 (5)	
Total Average:		48.6±1.6%	8.4±1.0%	7.8±2.3%

TABLE II:
CELL TYPE COMPOSITION OF DIFFERENTIATED CLONES
Subclones from Clones of Embryonic Hippocampal Precursor Cells

Subclone	Clone Size	MAP2+ (%)	GalC+ (%)	GFAP+ (%)
HI6.1	337	22 (7)	99 (29)	
HI6.2	338	13 (4)	157 (46)	
HI6.3	537	132 (25)	48 (9)	
HI6.4	565	98 (17)	28 (5)	
HI6.5	831	96 (12)	107 (13)	
HI6.6	886	158 (18)	134 (15)	
HI6.7	893	135 (15)	66 (7)	
HI6.8	950	154 (16)	53 (6)	
HI6.9	951	112 (12)	120 (13)	
HI6.10	970	105 (11)	95 (10)	
HI19.1	84	11 (13)		0 (0)
HI19.2	211	45 (21)		0 (0)
HI19.3	363	61 (17)		18 (5)
HI19.4	697	172 (25)		5 (1)
HI19.5	861	135 (16)		57 (7)
HI19.6	1469	401 (27)		123 (8)
HI19.7	1841	486 (26)		179 (10)
HI8.1	88		4 (5)	0 (0)
HI8.2	104		3 (3)	0 (0)
HI8.3	193		16 (8)	28 (15)
HI8.4	237		14 (6)	39 (16)
HI8.5	384		65 (17)	119 (31)
HI8.6	402		26 (6)	75 (19)
HI8.7	554		49 (9)	45 (8)
HI8.8	571		23 (4)	49 (9)
HI8.9	662		41 (6)	118 (18)
HI8.10	669		46 (7)	46 (7)
HI8.11	827		57 (7)	18 (2)
HI8.12	836		92 (11)	97 (12)
HI8.13	1084		104 (10)	53 (5)
HI8.14	1268		124 (10)	163 (13)
HI8.15	1284		75 (6)	193 (15)
Total Average:		20.1±1.4%	8.9±1.1%	10.0±0.7%

TABLE III:
CELL TYPE COMPOSITION OF DIFFERENTIATED CLONES
Clones of Adult Subependymal Cells

Passage	Clone Size	MAP2+ (%)	GalC+ (%)	GFAP+ (%)
1	73	6 (8)		37 (51)
1	159	56 (35)		42 (26)
1	173	57 (33)		26 (15)
1	185	71 (38)		32 (17)
1	230	97 (42)		39 (17)
1	273	139 (51)		56 (21)
1	387	117 (30)		45 (12)
1	554	237 (43)		84 (15)
1	675	280 (41)		74 (11)
1	847	399 (47)		155 (18)
1	496		23 (5)	92 (19)
1	526		7 (1)	115 (22)
1	644		19 (3)	26 (4)
1	713		22 (3)	179 (25)
1	1112		56 (5)	235 (21)
0	278	153 (55)	6 (2)	
0	305	145 (48)	19 (6)	
1	411	156 (38)	68 (17)	
0	513	242 (47)	3 (1)	
0	532	246 (46)	26 (5)	
0	538	283 (53)	10 (2)	
0	584	277 (47)	32 (5)	
0	1012	498 (49)	5 (0)	
Total Average:		41.7±2.6%	4.2±1.2%	19.6±2.7%

TABLE IV: EFFECT OF EXTRACELLULAR FACTORS ON CELL TYPE DETERMINATION

	(Antibody)	Untreated (%)	+PDGF (%)	+CNTF (%)	+T3 (%)
A. Embryonic					
Neuron	(MAP2)	45.9	81.0	0.9	11.5
Neuron	(TuJ1)	9.9	72.4	N.D.	N.D.
Neuron	(NF-M)	1.0	53.0	N.D.	N.D.
Oligodendrocyte	(GalC)	7.4	2.8	4.5	21.2
Astrocyte	(GFAP)	6.3	2.0	97.3	20.7
B. Adult					
Neuron	(MAP2)	36.8	73.9	11.8	35.2
Neuron	(TuJ1)	47.9	72.4	N.D.	N.D.
Oligodendrocyte	(GalC)	4.8	N.D.	N.D.	47.4
Astrocyte	(GFAP)	20.3	2.2	72.9	32.4

TABLE V: DIRECTED DIFFERENTIATION OF bFGF-EXPANDED HUMAN CNS STEM CELLS

(Antibody)	Untreated (%)	+PDGF (%)	+CNTF (%)	+T3 (%)
Neuron (MAP2+)	45.9±2.3	71.4±1.9	9.4±1.6	16.9±2.4
Oligodendrocytes (O4+/Galc+)	2.6±0.8	0.8±0.3	0.9±0.1	25.3±2.8
Astrocytes (GFAP+)	10.5±1.8	7.1±1.2	85.2±1.9	37.1±3.4
Dead	5.9±0.7	3.5±0.4	2.0±0.5	8.2±0.6

TABLE VI: NEUROTROPIC FACTORS AND DISEASES

Neurotropic factor:	**Disease**
Nerve growth factor:	Alzheimer's Disease Diabetic neuropathy Taxol neuropathy Compressive neuropathy AIDS-related neuropathy
Brain-derived growth factor:	Amyotrophic lateral sclerosis
Neurotrophin 3:	Large fiber neuropathy
Insulin-like growth factor:	Amyotrophic lateral sclerosis Vincristine neuropathy Taxol neuropathy
Ciliary neurotrophic factor:	Amyotrophic lateral sclerosis
Glia-derived neurotrophic factor:	Parkinson's Disease

TABLE VII: Proportions of Neuronal Phenotypes from Regionally Derived CNS Stem Cells[1]

Regions[2]	MAP2+[3]	% TH+	% AchE+	% VAT+	% ChAT+	% GAD+	% NPY+	% L-Enk+	% M-Enk+
SEPT	976±153	0.0	11.5±3.1	4.3±0.7	5.4±2.0	N.D.	0.0	0.0	0.0
LGE	2324±571	0.0	4.7±1.4	0.4±0.1	N.D.	N.D.	5.6±0.3	3.8±0.7	10.7±3.8
MGE	2414±518	0.0	6.0±1.8	0.4±0.1	N.D.	N.D.	2.8±0.1	5.1±2.5	5.0±0.6
HI	4286±1695	0.0	0.0	0.0	0.0	53.1±2.3	0.0	0.0	0.0
VM	2072±264	2.6±0.3	76.1±6.8	0.0	N.D.	84.4±6.5	N.D.	N.D.	N.D.
DM	1385±95	0.0	100.9±9.1	0.0	N.D.	N.D.	N.D.	N.D.	N.D.
SPC	1529±18	0.0	39.3±2.5	1.2±0.1	N.D.	N.D.	N.D.	N.D.	N.D

TABLE VII LEGEND:

[1]Numbers for different neuronal phenotypes are given as the percentage of MAP2ab-positive neurons per square centimeter for each region. MAP2, microtubule associated protein a and b; TH, tyrosine hydroxylase; AchE, acetylcholine esterase; VAT, vesicular acetylcholine transporter; ChAT, choline acetyl transferase; GAD, glutamic acid decarboxylase; NPY, neuropeptide Y; L-Enk, leu-enkephalin; M-Enk, met-enkephalin.

[2]Regions from where CNS stem cells were derived. SEPT, E15.5 septum; LGE, E15.5 lateral ganglionic eminence; MGE, E15.5 medial ganglionic eminence; HI, E15.5 hippocampus; VM, E12.5 ventral mesencephalon; DM, E12.5 dorsal mesencephalon; SPC, E13.5 spinal cord.

[3]Shown are the average number of MAP2ab-positve cells per square centimeter. Initial number of cells plated for all regions was 125,000 cells per square centimeter. ± standard mean error.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for expansion and long-term culture *in vitro* of stem cells of the central nervous system of a mammal, wherein the stem cells maintain the multipotential capacity to differentiate into neurons, astrocytes, and oligodendrocytes, comprising the steps of:

a) dissociating cells from central nervous system tissue by mechanical trituration;

b) culturing the dissociated cells adhered onto a plate in a chemically defined serum-free culture medium;

c) plating dissociated cells at a density not exceeding 20,000 cells/cm^2 and, in subsequent passages, replating the cultured cells at a density not exceeding 10,000 cells/cm^2;

d) adding daily to the cultured cells a growth factor selected from the group consisting of

i) bFGF at a concentration of at least 10 ng/ml,

ii) EGF at a concentration of at least 10 ng/ml,

iii) TGF-alpha at a concentration of at least 10 ng/ml, and

iv) aFGF at a concentration of at

least 10 ng/ml plus 1 μg/ml heparin;

e) replacing the culture medium with fresh medium within every two days;

f) passaging the cultured cells within four days after plating so as not to exceed 50% confluence; and

g) passaging the cultured cells by treating the cultured cells with saline solution and scraping cells from the plate.

2. The method of claim 1, wherein the stem cells are derived from central nervous system tissue from a human.

3. The method of claim 2, wherein the stem cells are derived from central nervous system tissue from a fetus.

4. The method of claim 3, wherein the stem cells are derived from central nervous system tissue selected from the group consisting of hippocampus, cerebral cortex, striatum, septum, diencephalon, mesencephalon, hindbrain, and spinal cord.

5. The method of claim 2, wherein the stem cells are derived from central nervous system tissue from an adult.

6. An *in vitro* culture of stem cells of the central nervous system of a mammal, wherein the stem cells maintain the multipotential capacity to differentiate into neurons, astrocytes, and oligodendrocytes.

7. The *in vitro* culture of claim 6, wherein the stem cells are derived from central nervous system tissue from a human.

8. The *in vitro* culture of claim 7, wherein the stem cells are derived from central nervous system tissue from a fetus.

9. The *in vitro* culture of claim 8, wherein the stem cells are derived from central nervous system tissue selected from the group consisting of hippocampus, cerebral cortex, striatum, septum, diencephalon, mesencephalon, hindbrain, and spinal cord.

10. The *in vitro* culture of claim 7, wherein the stem cells are derived from central nervous system tissue from an adult.

11. The *in vitro* culture of claim 10, wherein the stem cells are derived from central nervous system tissue selected from the group consisting of

hippocampus, cerebral cortex, striatum, septum, diencephalon, mesencephalon, hindbrain, and spinal cord.

12. The *in vitro* culture of claim 6, wherein the stem cells differentiate to mature neurons exhibiting axon-dendrite polarity, synaptic terminals, and localization of proteins involved in synaptogenesis and synaptic activity including neurotransmitter receptors, transporters, and processing enzymes.

13. The *in vitro* culture of claim 6, wherein the stem cells retain their capacity to generate subtypes of neurons having molecular differences among the subtypes.

14. A method for differentiation of an *in vitro* culture of stem cells of the central nervous system of a mammal, wherein the stem cells maintain the multipotential capacity to differentiate into neurons, astrocytes, and oligodendrocytes, comprising the steps of:

a) dissociating cells from central nervous system tissue by mechanical trituration;

b) culturing the dissociated cells on a plate in complete absence of serum;

c) adding daily to the cultured cells a

first growth factor selected from the group consisting of

i) bFGF at a concentration of at least 10 ng/ml,

ii) EGF at a concentration of at least 10 ng/ml,

iii) TGF-alpha at a concentration of at least 10 ng/ml, and

iv) aFGF at a concentration of at least 10 ng/ml plus 1 μg/ml heparin;

d) passaging the cultured cells by treating the cultured cells with saline solution and scraping the cells from the plate; and

e) removing the first growth factor from the cultured cells.

15. The method of claim 14, further comprising adding a second growth factor to the cultured cells after removing the first growth factor from the cultured cells.

16. The method of claim 15, wherein the second growth factor is selected from the group consisting of platelet-derived growth factor (PDGF), ciliary neurotropic factor (CNTF), leukemia inhibitory factor (LIF), and thyroid hormone, iodothyronine (T3).

17. A method for *in vitro* generation of region- specific, terminally differentiated, mature neurons from cultures of mammalian multipotential CNS stem cells, comprising the steps of:

a) culturing multipotential CNS stem cells from a specific region in a chemically defined serum-free culture medium containing a growth factor;

b) replacing the medium with growth factor-free medium;

c) harvesting the stem cells by trypsinization;

d) plating the stem cells at a density of between 100,000 to 250,000 cells per square centimeter; and

e) culturing the cells in a glutamic acid-free chemically defined serum-free culture medium.

18. The method of claim 17, wherein said specific region is selected from the group consisting of cortex, olfactory tubercle, retina, septum, lateral ganglionic eminence, medial ganglionic eminence, amygdala, hippocampus, thalamus, hypothalamus, ventral and dorsal mesencephalon, brain stem, cerebellum, and spinal cord.

19. The method of claim 17, wherein said

chemically defined serum-free culture medium is N2 or N2-modified media.

20. The method of claim 17, wherein the growth factor is selected from the group consisting of bFGF, EGF, TGF-alpha and aFGF.

21. The method of claim 17, further comprising culturing the cells in a glutamic acid-free chemically defined serum-free culture medium supplemented with between 10-100 ng/ml of brain-derived neurotropic factor.

22. The method of claim 17, wherein the mammalian multipotential CNS stem cells are derived from central nervous system tissue from a rat.

23. The method of claim 17, wherein the mammalian multipotential CNS stem cells are derived from central nervous system tissue from a human.

24. An *in vitro* culture of region-specific, terminally differentiated, mature neurons derived from cultures of mammalian multipotential CNS stem cells from a specific region.

25. The *in vitro* culture of claim 24, wherein said specific region is selected from the group

consisting of cortex, olfactory tubercle, retina, septum, lateral ganglionic eminence, medial ganglionic eminence, amygdala, hippocampus, thalamus, hypothalamus, ventral and dorsal mesencephalon, brain stem, cerebellum, and spinal cord.

26. The *in vitro* culture of claim 24, wherein the stem cells are derived from central nervous system tissue from a rat.

27. The *in vitro* culture of claim 24, wherein the stem cells are derived from central nervous system tissue from a human.

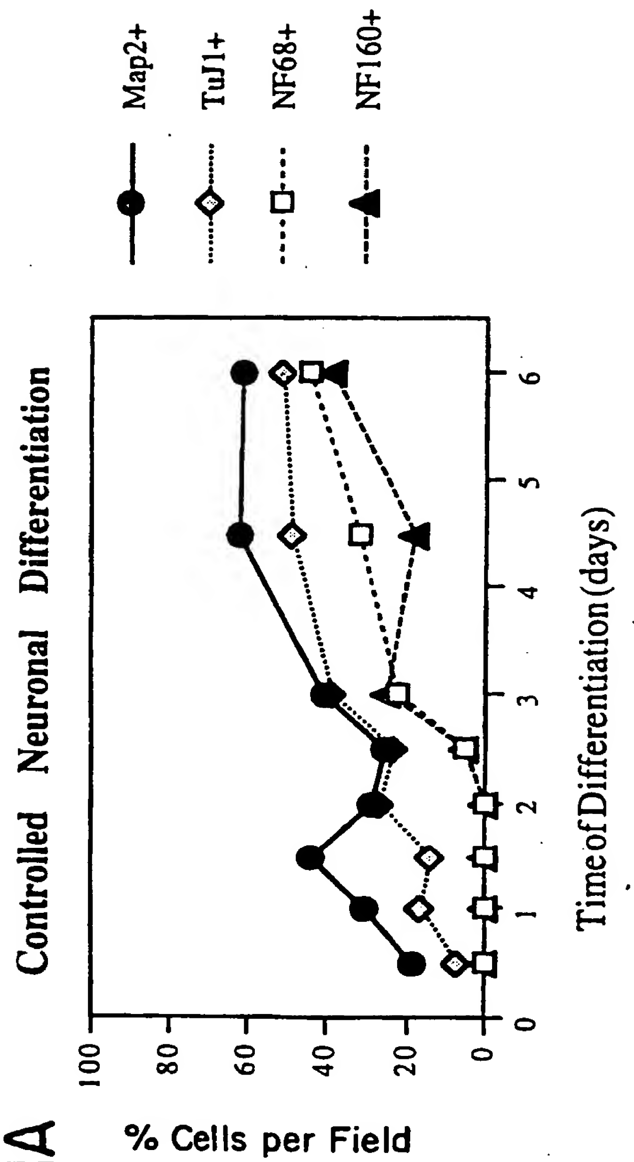

Controlled Neuronal Differentiation
Map2+
TuJ1+
NF68+
NF160+
% Cells per Field
100
80
60
40
20
0
0
1
2
3
4
5
6
Time of Differentiation (days)

FIG. 1A

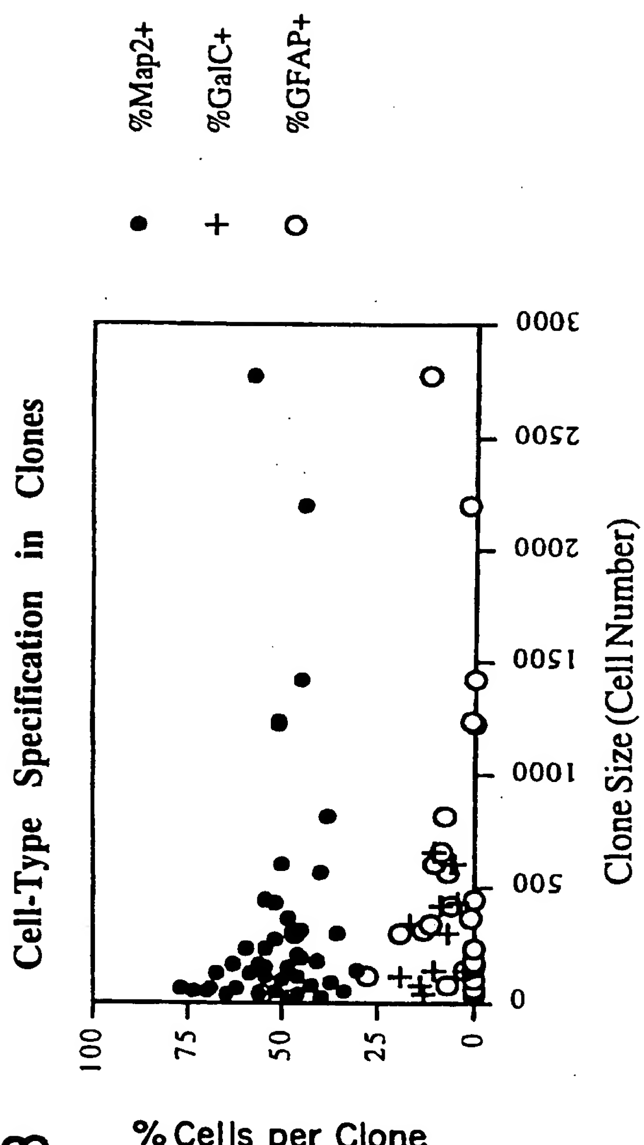

Cell-Type Specification in Clones
%Map2+
%GalC+
%GFAP+
% Cells per Clone
100
75
50
25
0
0
500
1000
1500
2000
2500
3000
Clone Size (Cell Number)

FIG. IB

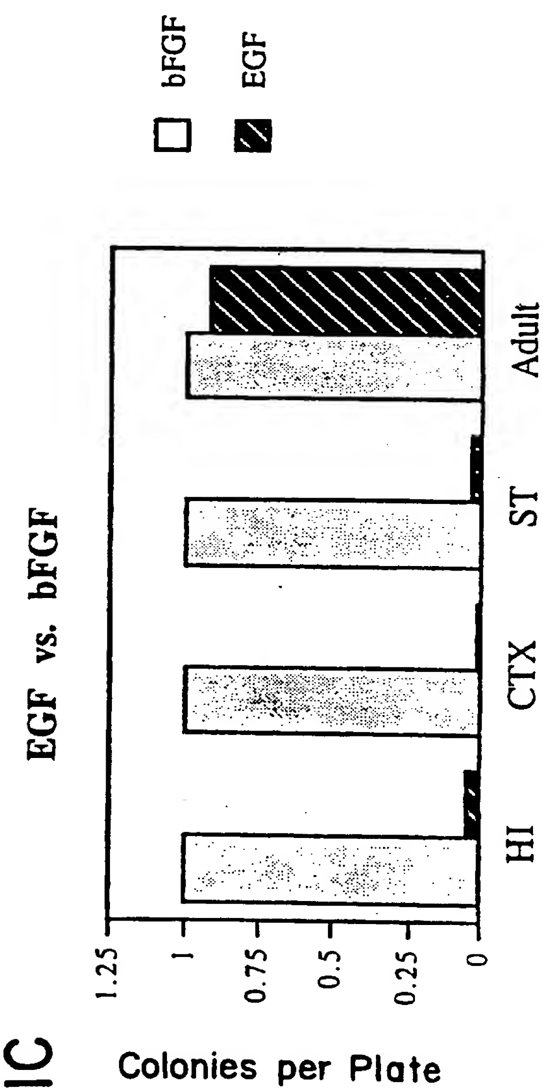

EGF vs. bFGF
bFGF
EGF
Colonies per Plate
1.25
1
0.75
0.5
0.25
0
HI
CTX
ST
Adult

FIG. IC

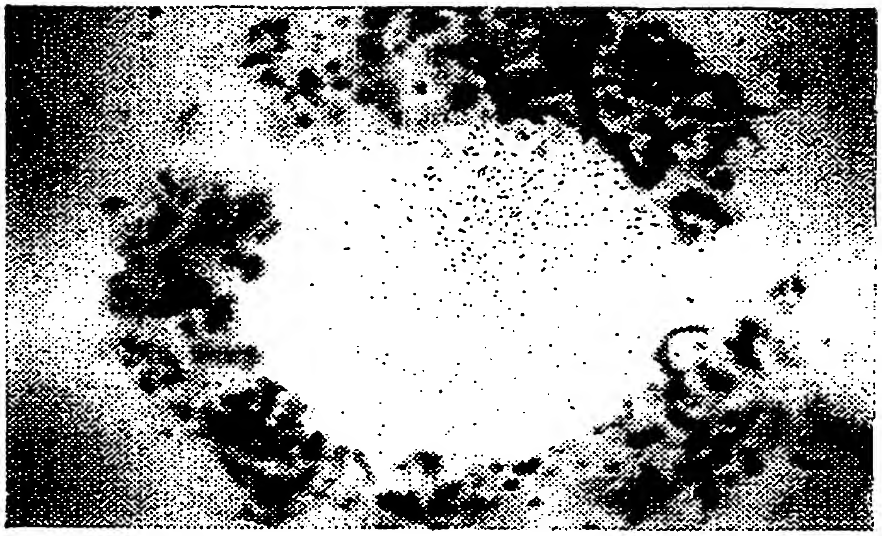


FIG. 2A

FIG. 2B

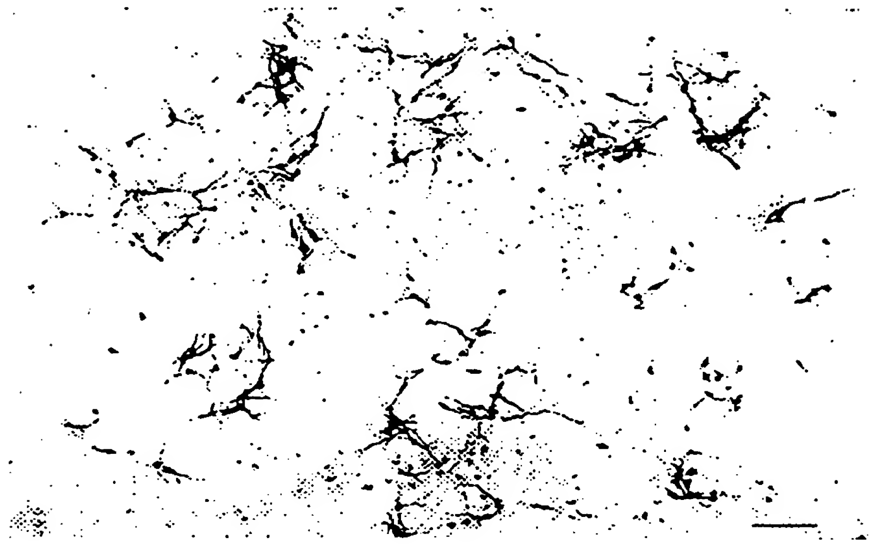


FIG. 2C

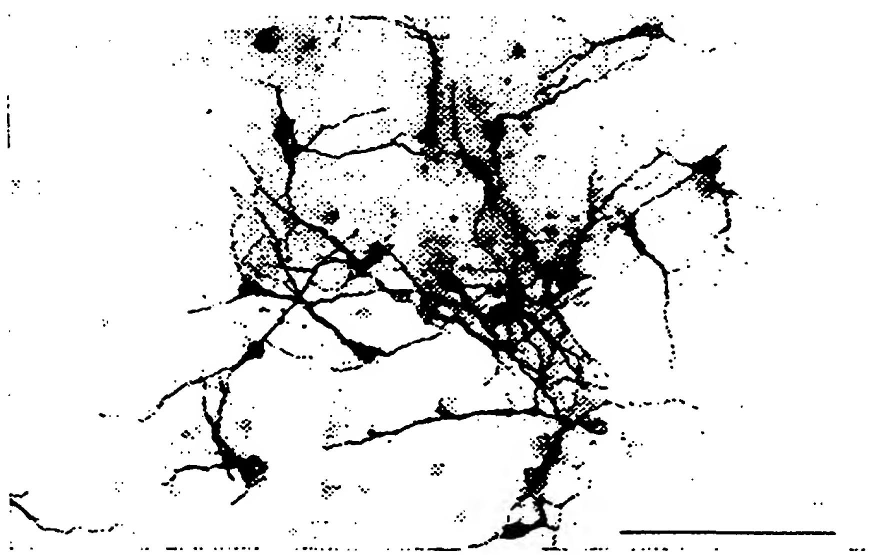


FIG. 2D

FIG. 3B FIG. 3D FIG. 3F

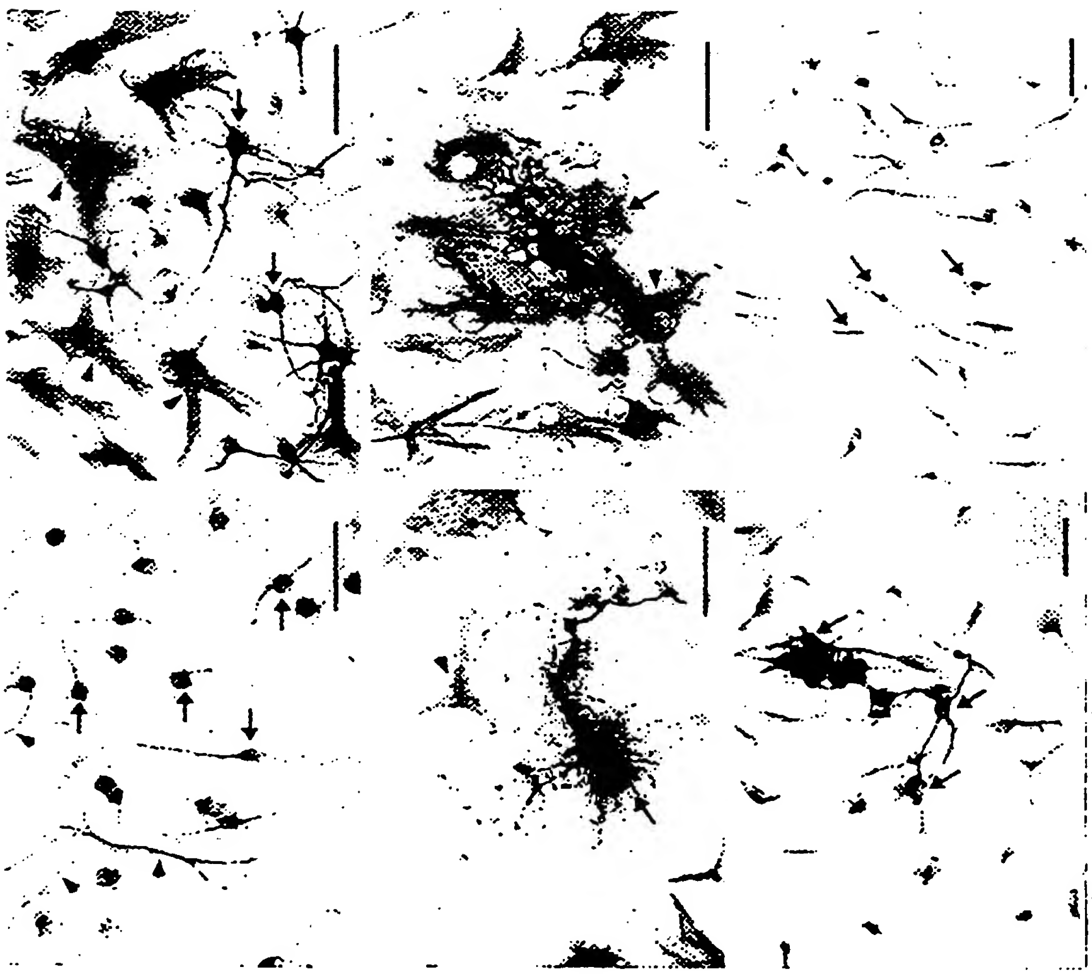


FIG. 3A FIG. 3C FIG. 3E

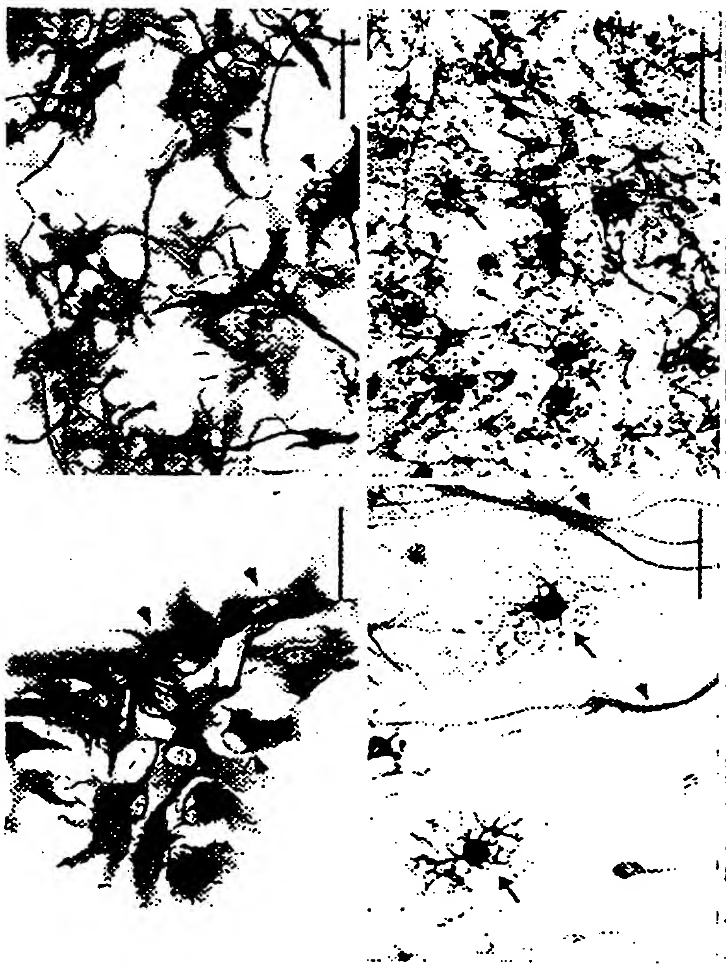


FIG. 3G

FIG. 3H

FIG. 3I

FIG. 3J

FIG. 4

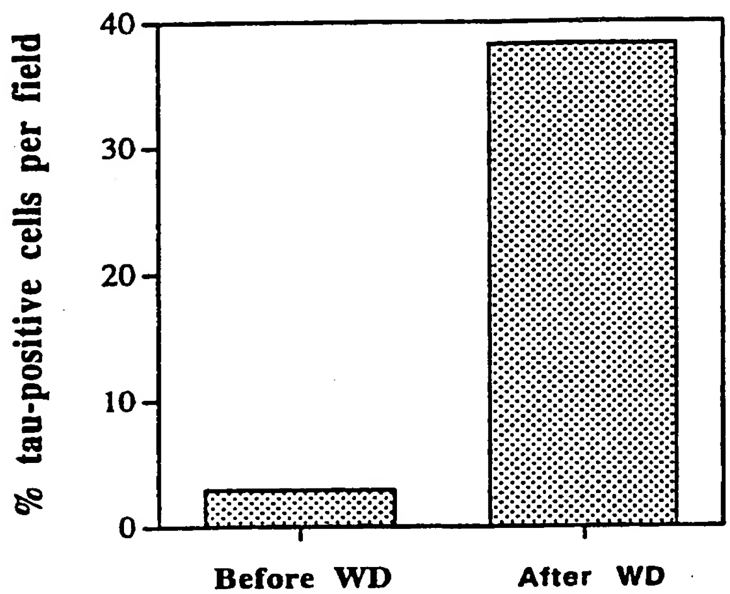


FIG. 5B

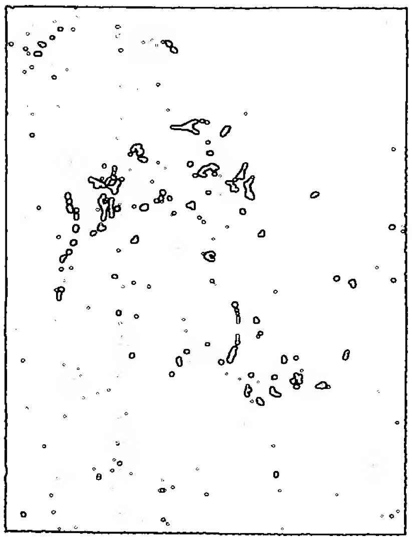


FIG. 5D

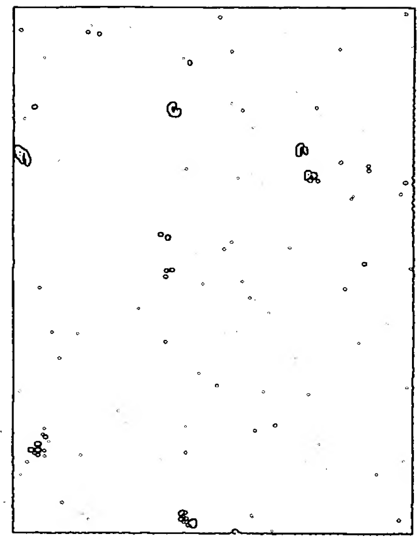
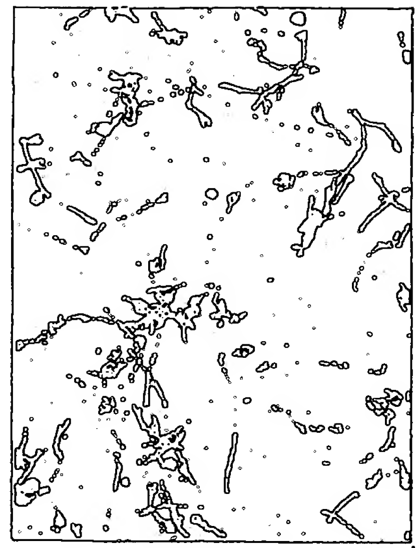


FIG. 5F



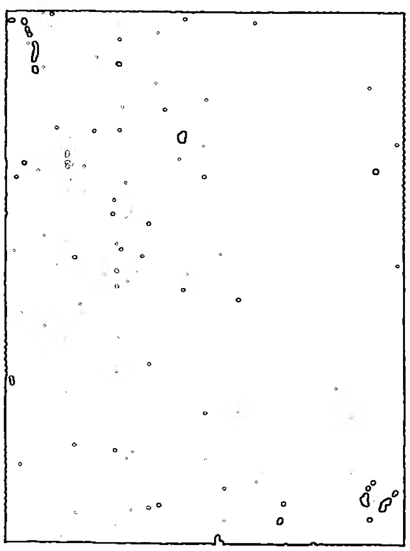


FIG. 5A

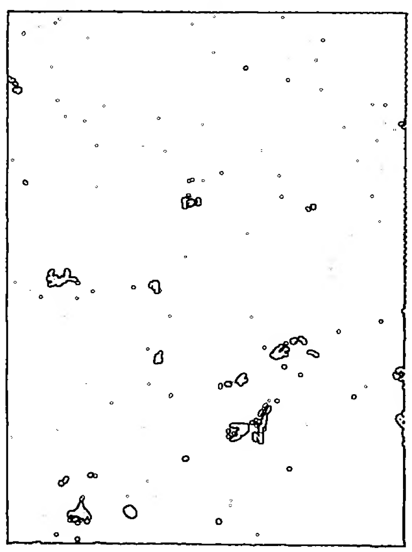


FIG. 5C

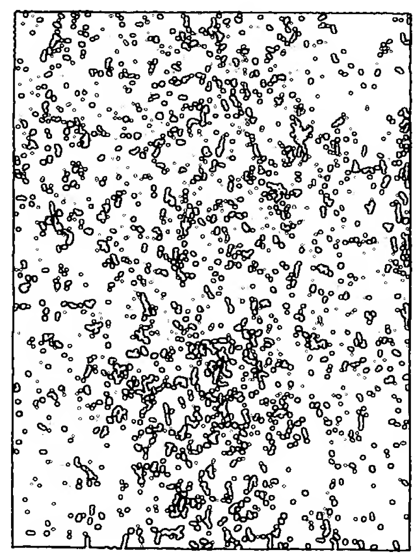


FIG. 5E

FIG. 6A

FIG. 6B

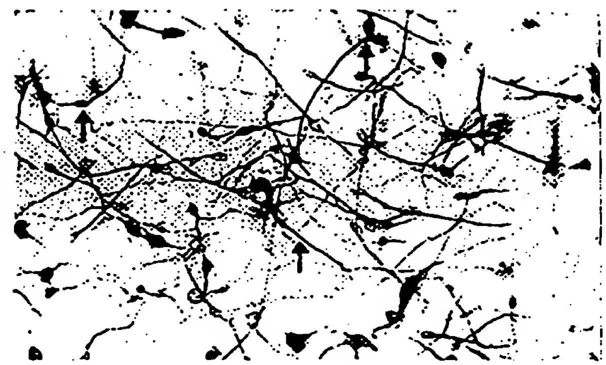


FIG. 6C

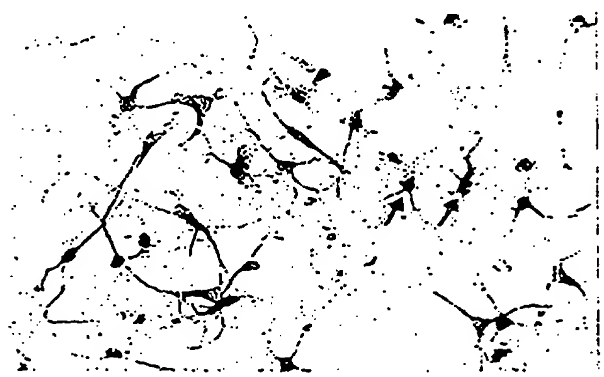


FIG. 6D

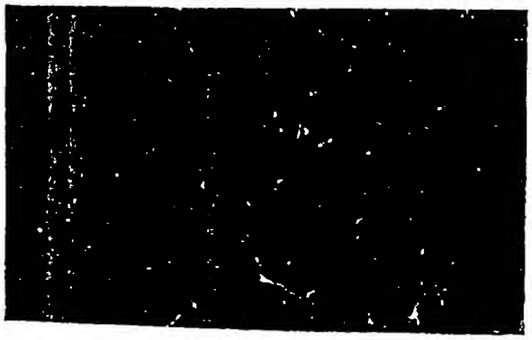


FIG. 7A

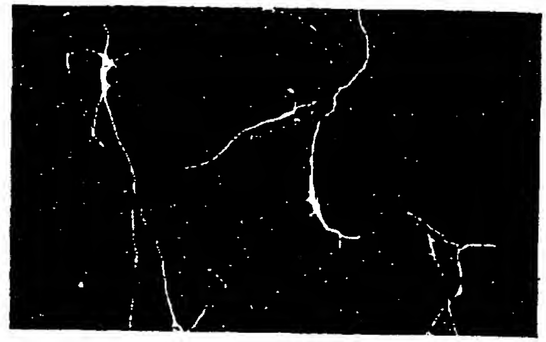


FIG. 7B

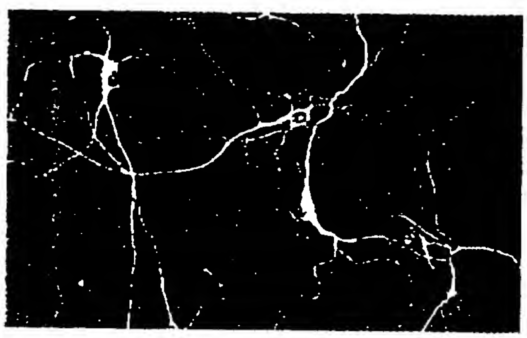


FIG. 7C

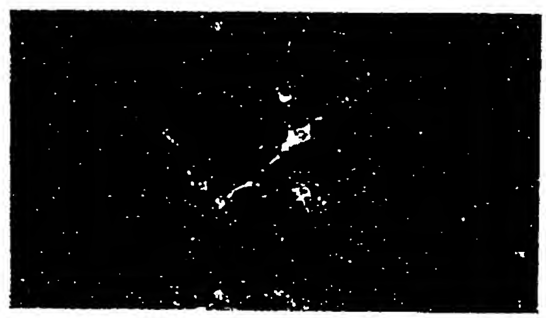


FIG. 7D

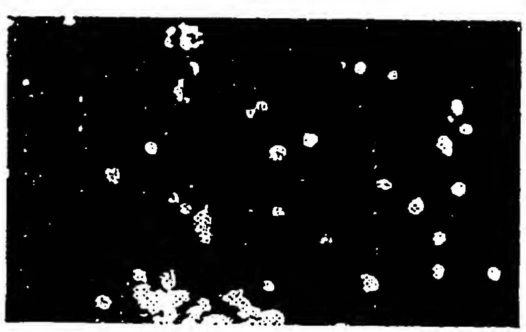


FIG. 7E

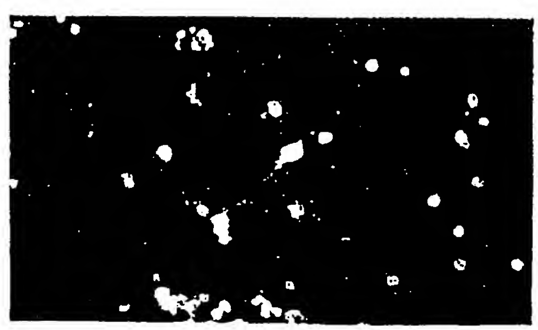


FIG. 7F

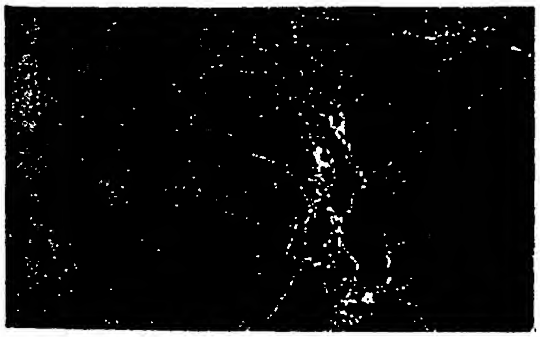


FIG. 7G

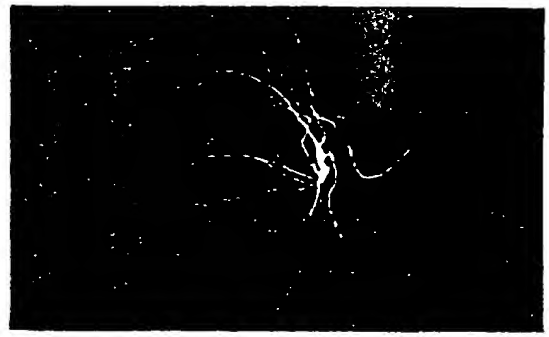


FIG. 7H

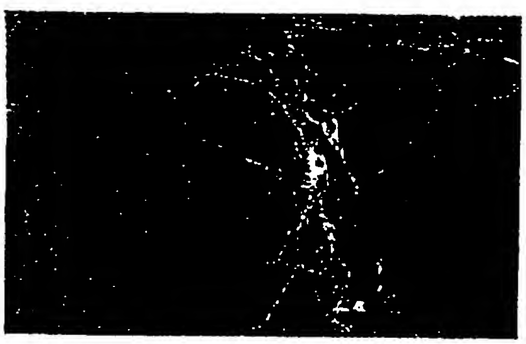


FIG. 7I

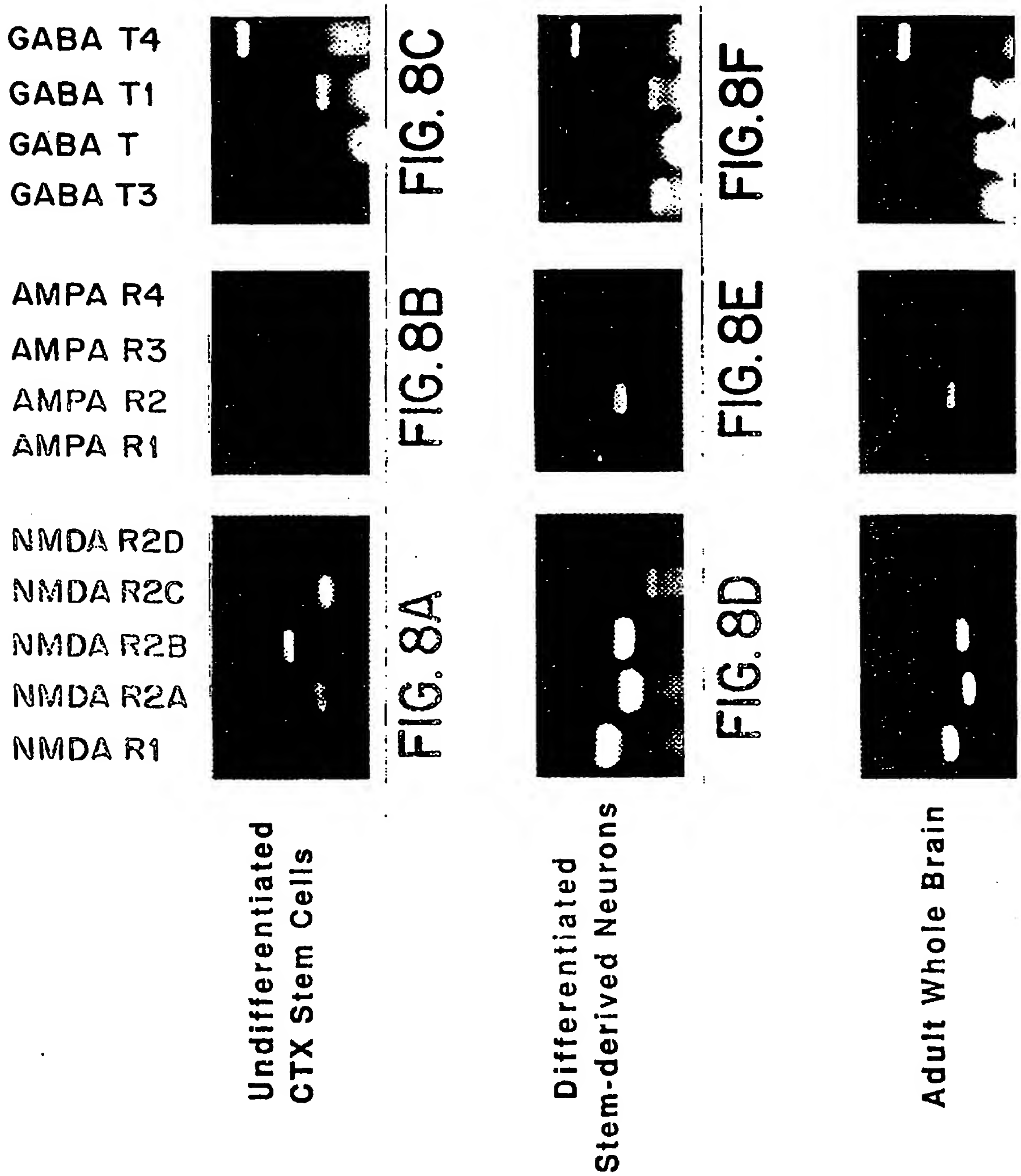


FIG. 8A FIG. 8B FIG. 8C

FIG. 8D FIG. 8E FIG. 8F

FIG. 8G FIG. 8H FIG. 8I

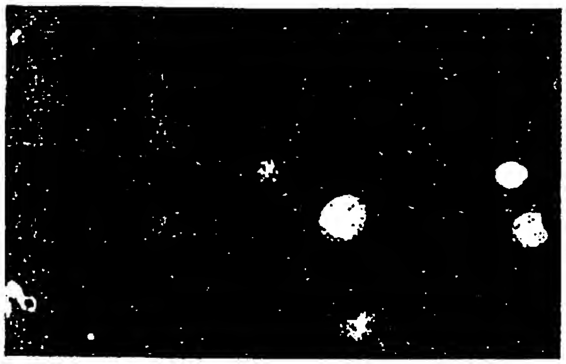


FIG. 9A

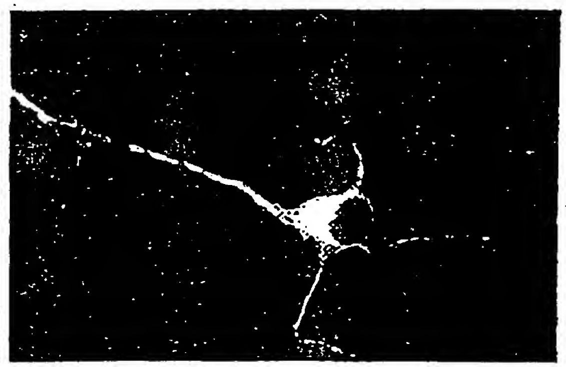


FIG. 9B

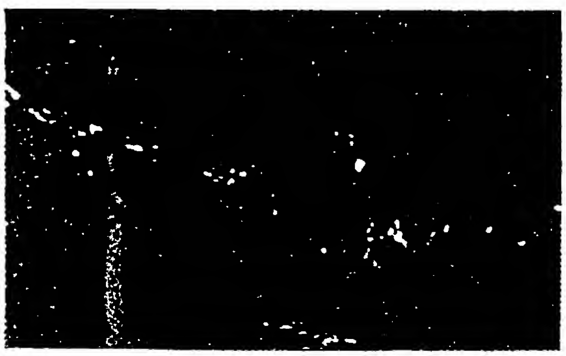


FIG. 9C

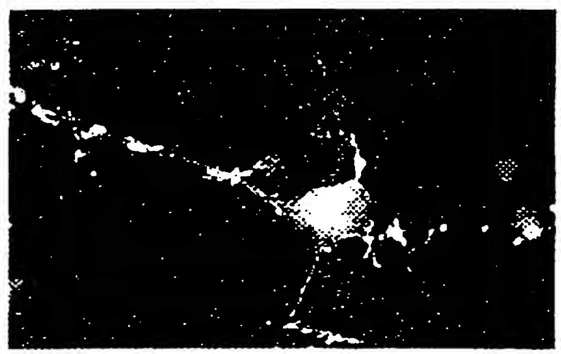


FIG. 9D

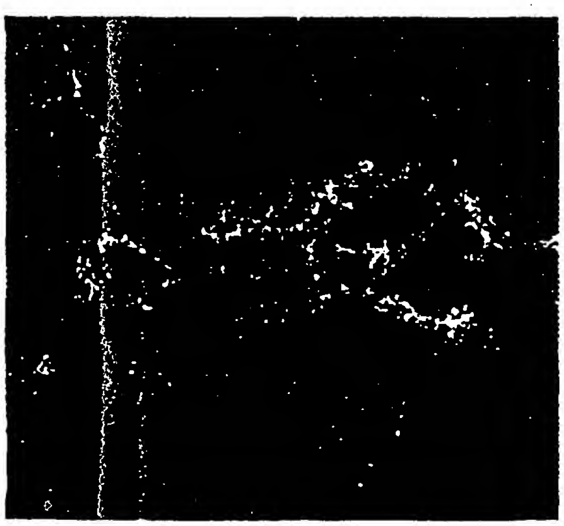


FIG. 9E

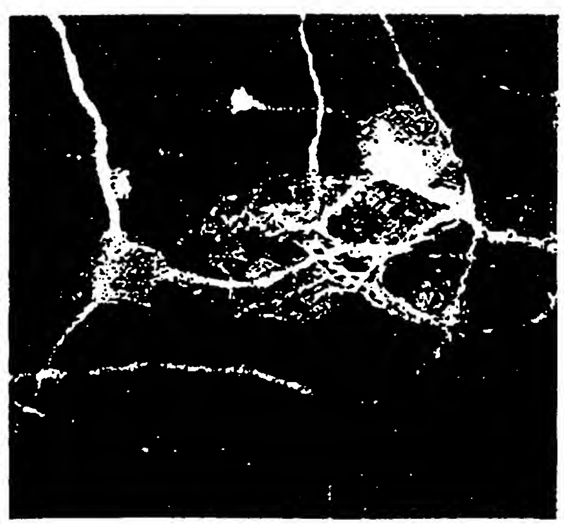


FIG. 9F

FIG. 10

FIG. 11A

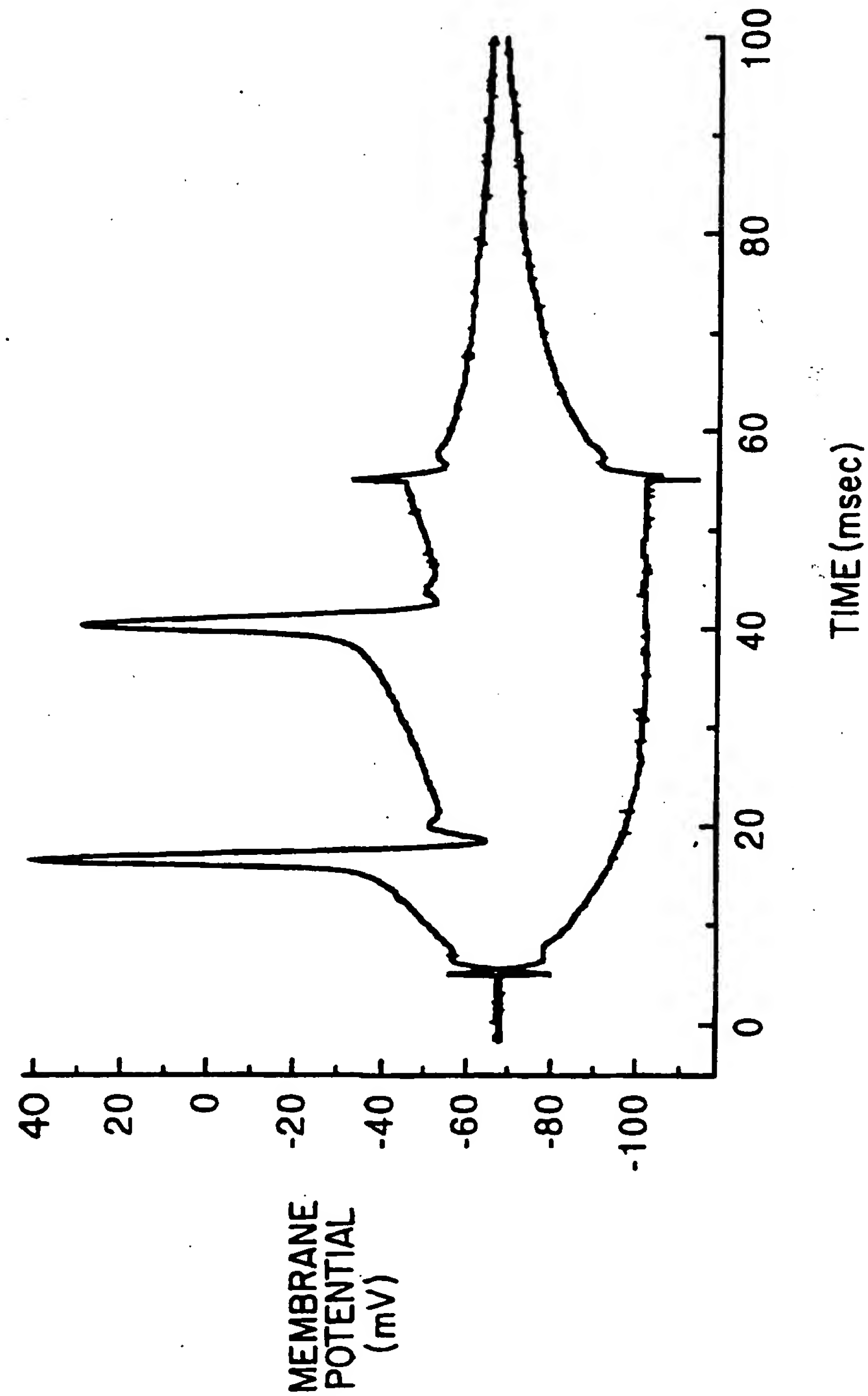

40
20
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
0
20
40
60
80
100
TIME (msec)

FIG. 11B

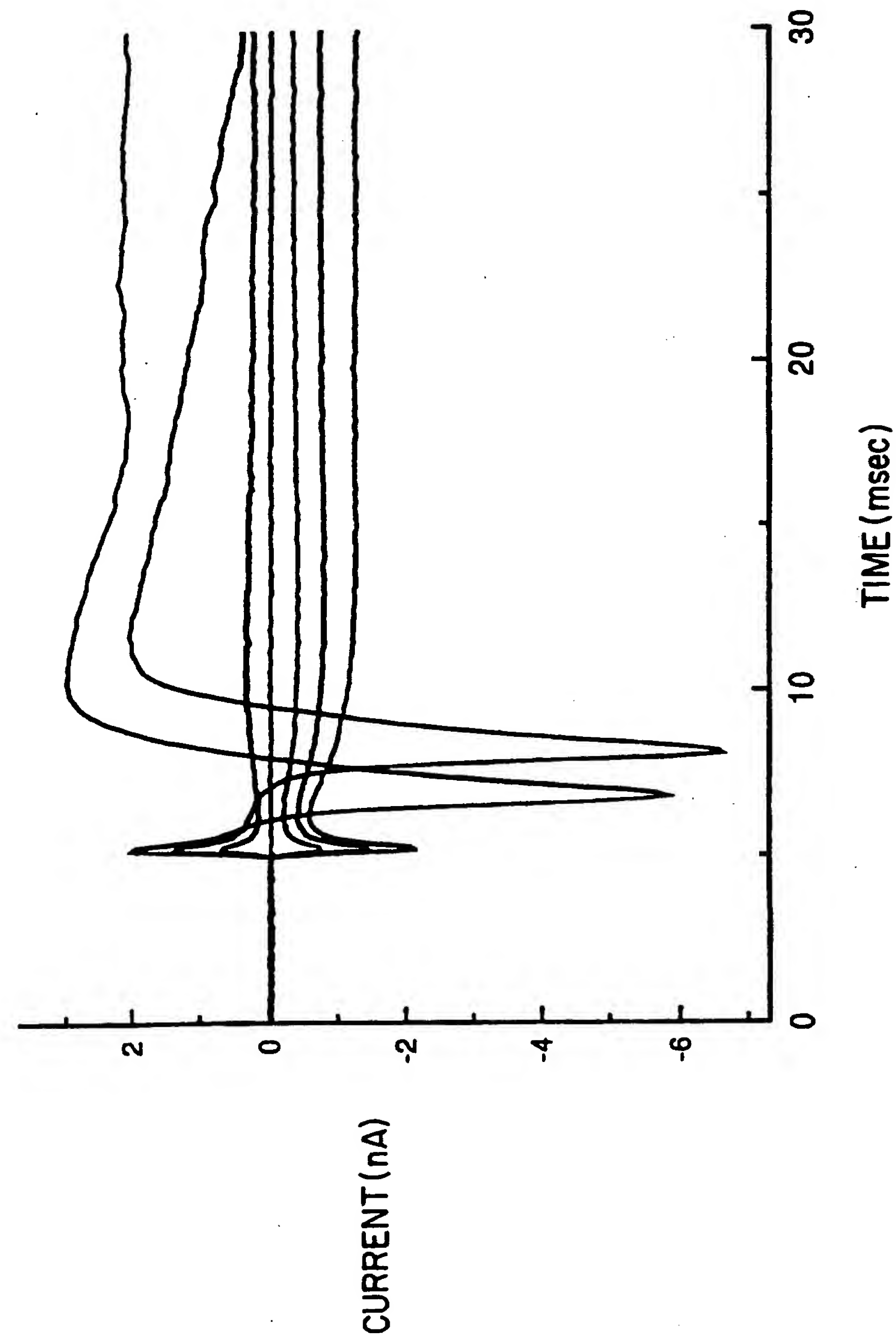


FIG. 11C

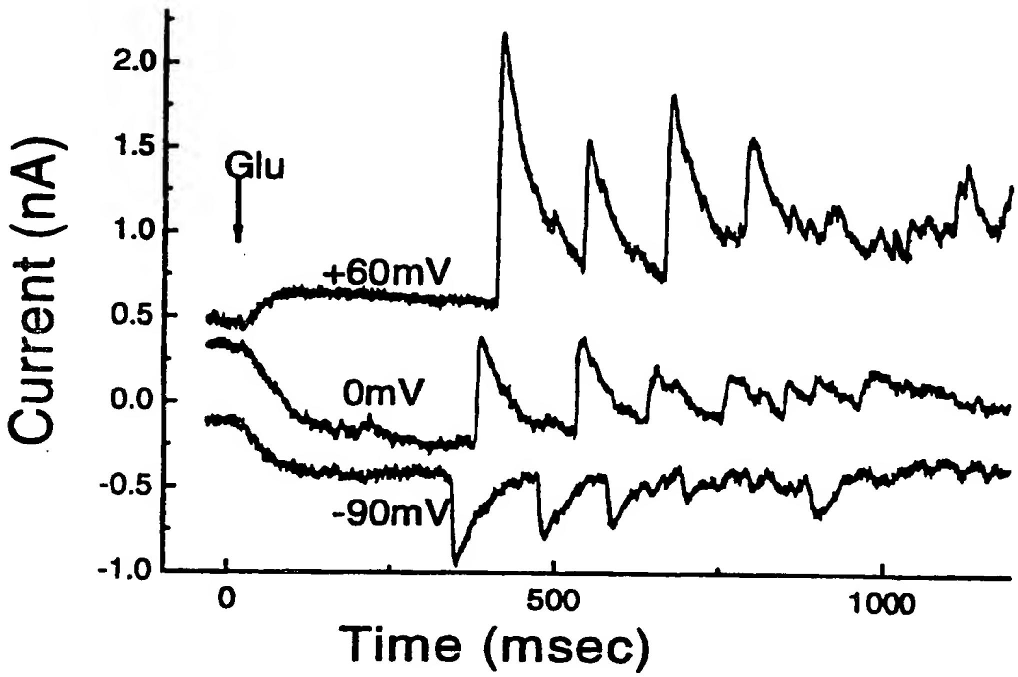
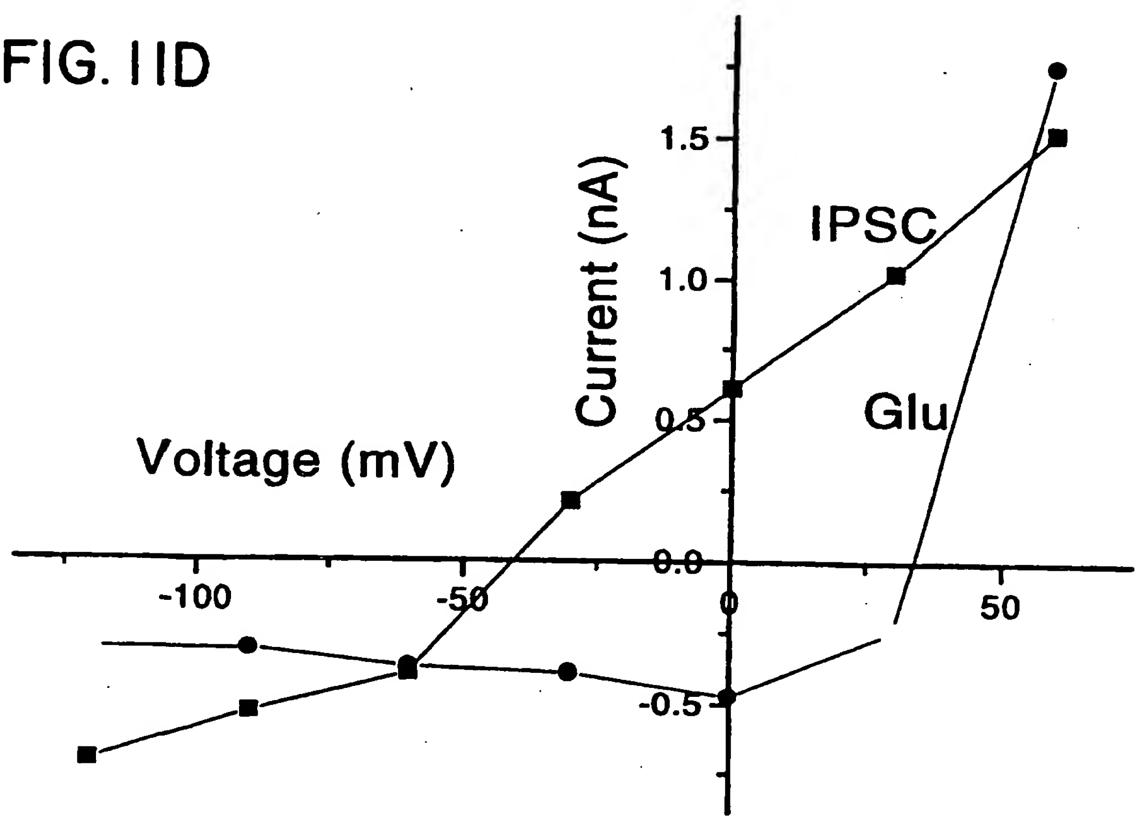


FIG. 11D



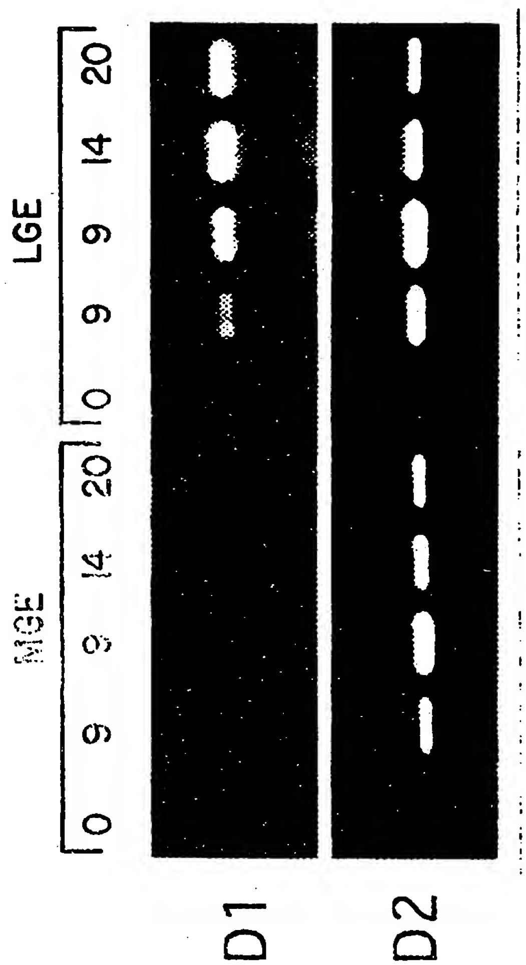


FIG.12A

FIG.12B

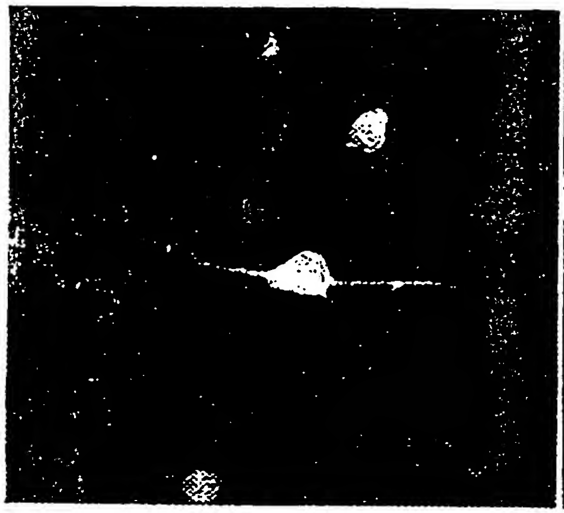


FIG. 13A

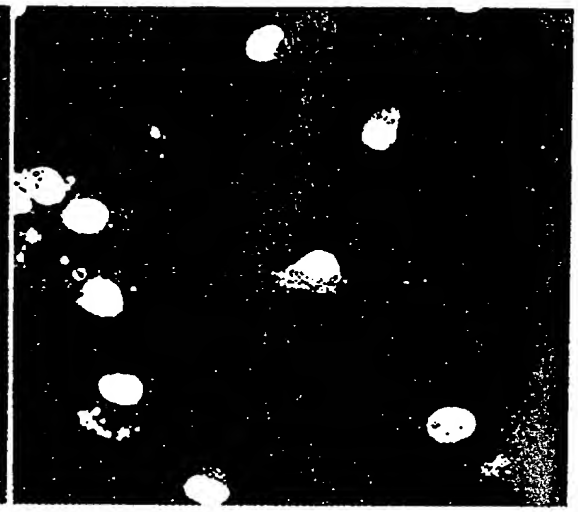


FIG. 13B

FIG. 13C

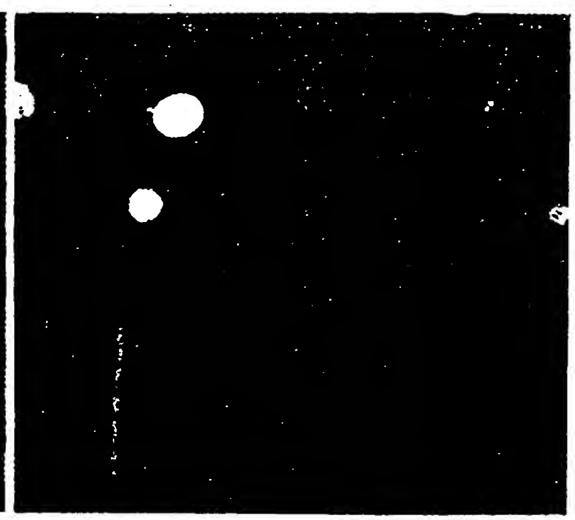


FIG. 13D

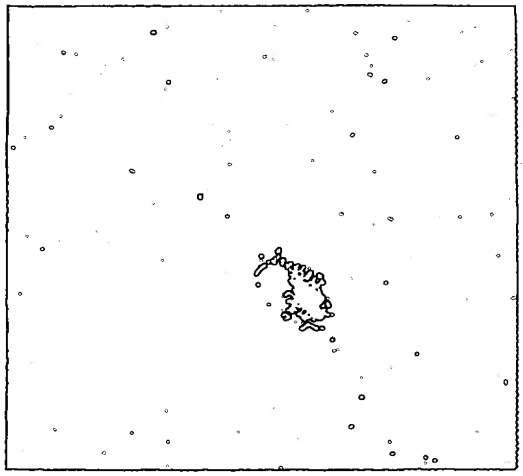


FIG. 14A

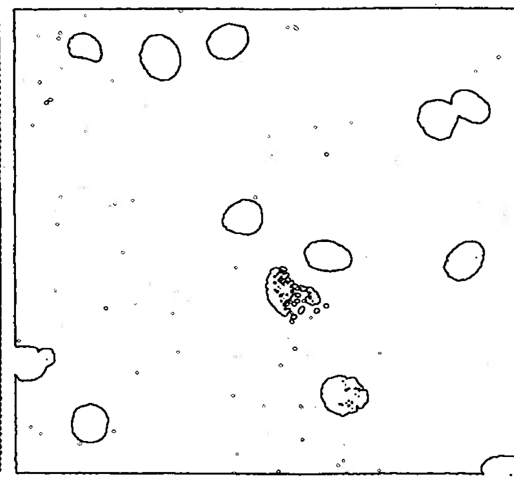


FIG. 14B

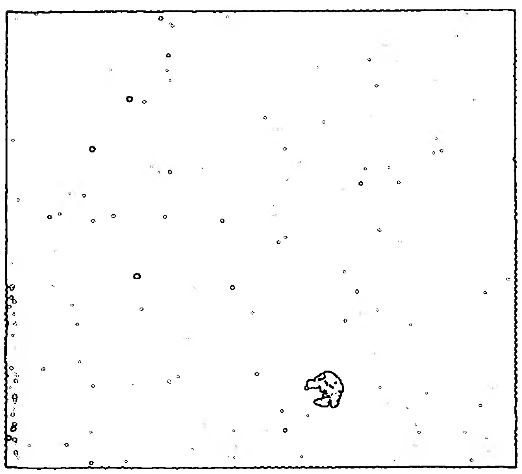


FIG. 14C

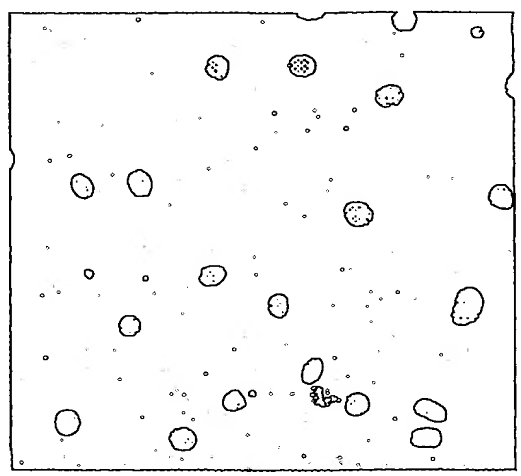


FIG. 14D

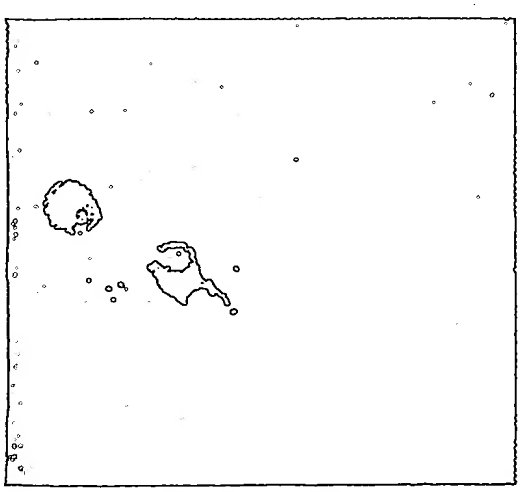


FIG. 14E

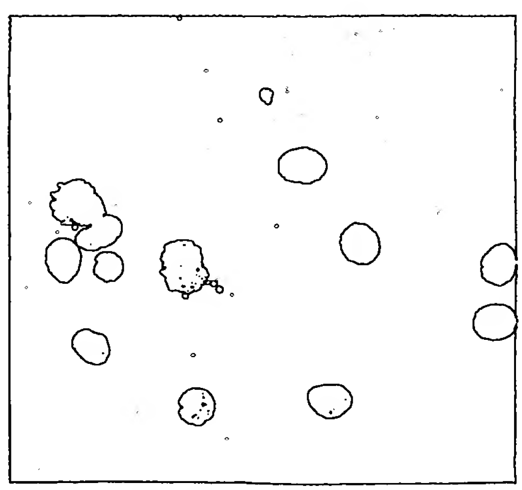


FIG. 14F

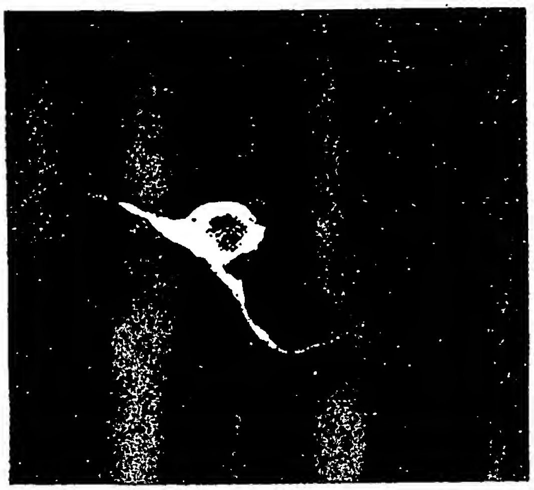


FIG. 15A

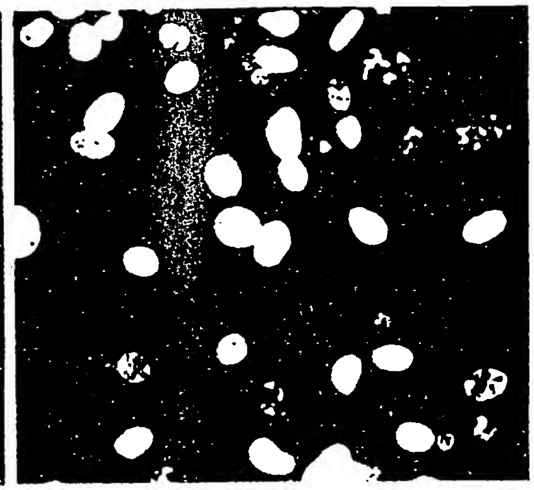


FIG. 15B

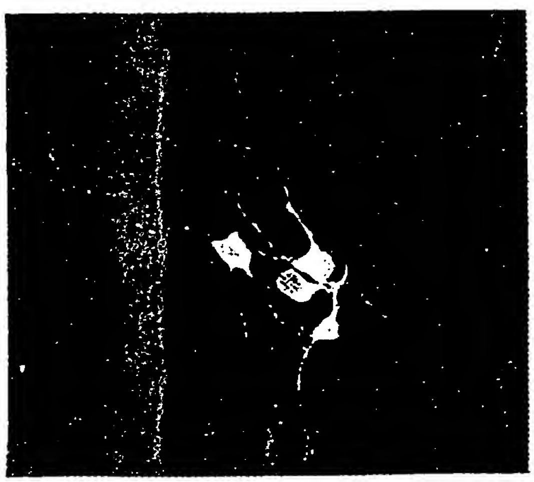


FIG. 15C

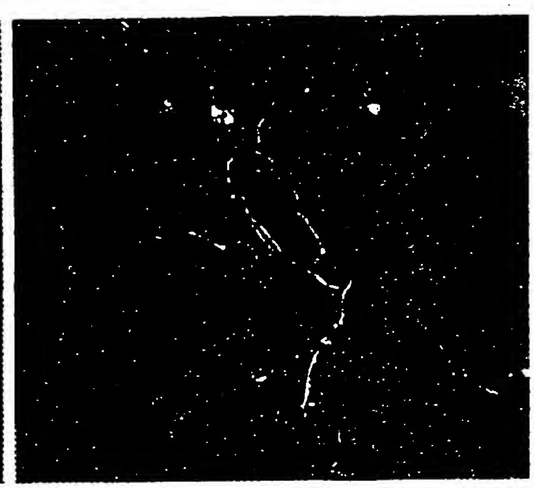


FIG. 15D

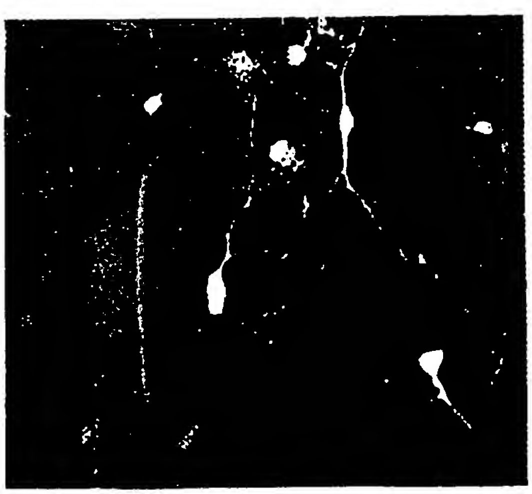


FIG. 15E

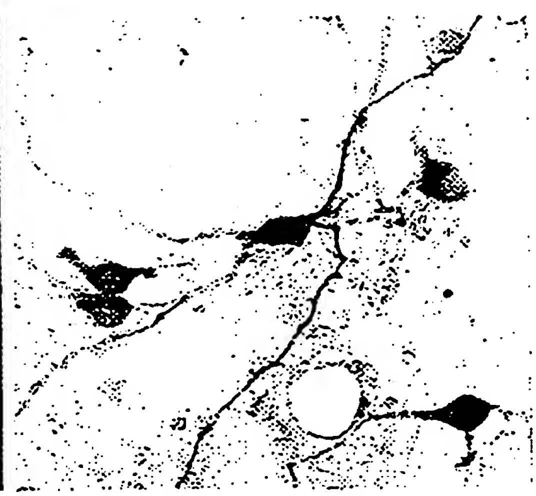


FIG. 15F

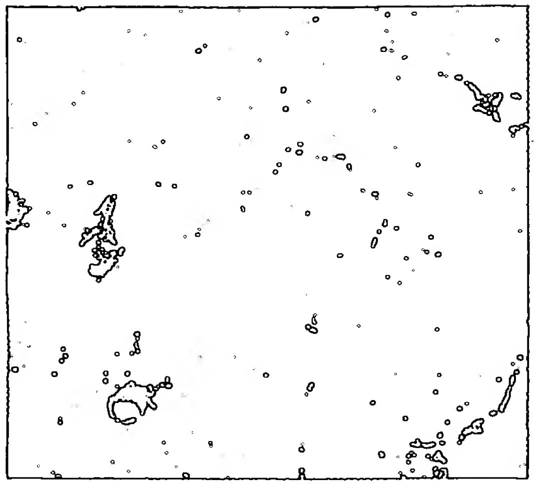


FIG. 18A

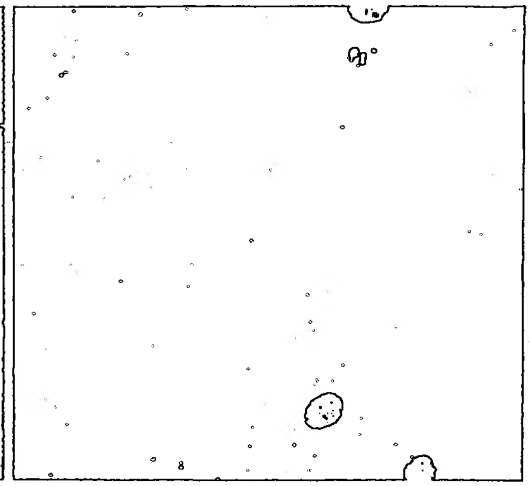


FIG. 18B

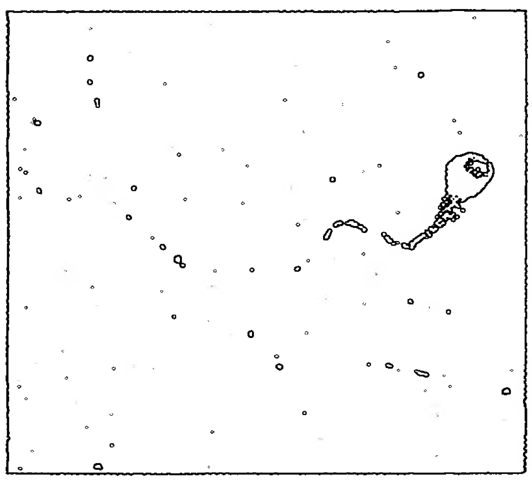


FIG. 18C

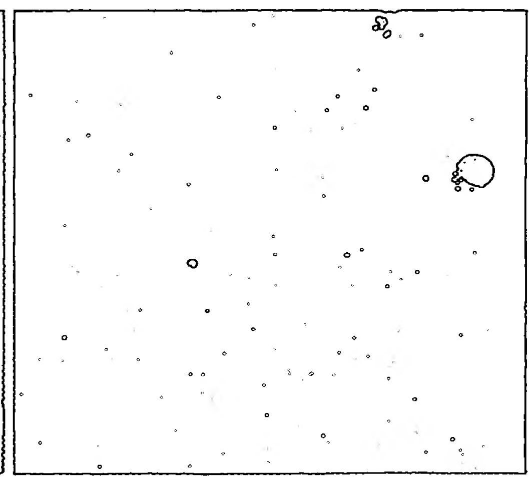


FIG. 18D

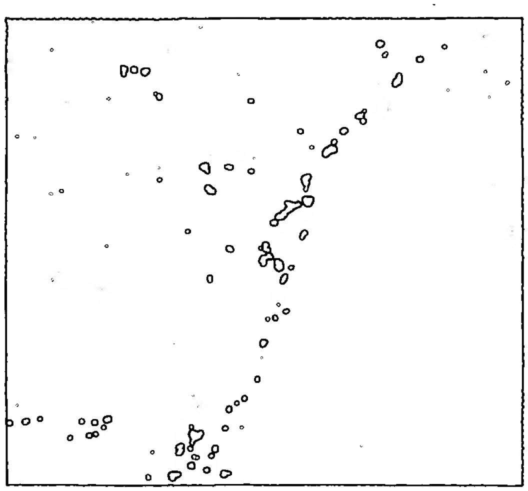


FIG. 18E

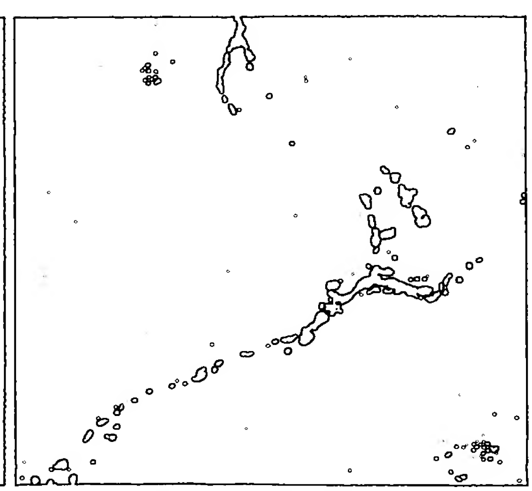


FIG. 18F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/07669

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 10292 A (NEUROSPHERES LTD.) 11 May 1994 see claims; examples 1-3 ---	1-27
A	WO 96 09543 A (NEUROSPHERES LTD.) 28 March 1996 see claims; examples 1-3 ---	1-27
A	EXPERIMENTAL NEUROLOGY, vol. 128, 1994, NEW YORK, N.Y., US, pages 34-40, XP002037766 J.R. VON VISGER ET AL.: "DIFFERENTIATION AND MATURATION OF ASTROCYTES DERIVED FROM NEUROEPITHELIAL PROGENITOR CELLS IN CULTURE." see the whole document --- -/--	1-27

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
14 August 1997	11. 09. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

INTERNATIONAL SEARCH REPORT

Int[...]onal Application No
PCT/US 97/07669

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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